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(57) Abstract

Disclosed are peptide–based compositions and methods for inhibiting and modulating the actions of CXC intercrine molecules. The antileukinate peptides described inhibit IL–8, GRO and MIP2β binding to neutrophils and neutrophil activation. The peptides are particularly advantageous as they inhibit IL–8–induced enzyme release at a 25 fold lower concentration than is required to inhibit chemotaxis, which makes them ideal for treating various inflammatory diseases and disorders including, amongst others, Adult Respiratory Distress Syndrome (ARDS), cystic fibrosis and chronic bronchitis. The invention further includes methods for inhibiting tumor cell growth by employing selected members of the disclosed group of peptides to inhibit α–chemokine binding to the tumor cell.

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DESCRIPTION

PEPTIDE COMPOSITIONS FOR TUMOR INHIBITION

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention concerns methods and compositions for inhibiting and modulating the actions of CXC intercrine molecules, particularly for inhibition of tumor cell proliferation. Disclosed are peptide compositions which inhibit interleukin 8 (IL-8) and, particularly, which preferentially inhibit IL-8-induced release of degradative enzymes by neutrophils. These compositions may be employed to treat conditions in which it is desirable to inhibit tumor cell growth.

2. Description of the Related Art

IL-8 is a member of the CXC intercrine family of cytokines, so named due to elements of their N-terminal sequences. This family also includes, amongst others, peptide molecules known as growth related oncogene (GRO, or GRO/MGSA) and macrophage inflammatory protein 2β (MIP2β). IL-8 is a peptide of approximately 8 kD, and is about 72 amino acids in length, with this length varying according to the post-translational processing in different cell types (Yoshimura *et al.*, 1989; Hebert *et al.*, 1990; Strieter *et al.*, 1989). The IL-8 gene was first identified by analyzing the genes transcribed by human blood mononuclear cells stimulated with Staphylococcal enterotoxin A (Schmid and Weissman, 1987). IL-8 production is known to be induced by tumor necrosis factor and interleukin 1 (Strieter *et al.*, 1990).

MGSA/GROα is a peptide which was first identified as an autostimulatory growth factor of Hs 294T melanoma cells (Richmond *et al.*, 1983; 1985; Richmond and Thomas, 1986). Further studies indicated that it was produced by diverse melanoma cell lines (Lawson *et al.*, 1987) and played an important role in the tumorigenesis and growth of malignant melanoma cells (Mintz and Silvers, 1993). Sequence analysis demonstrated that MSGA/GROα is a member of the superfamily called "α-chemokines" (Oppenheim *et al.*, 1991). This family of proteins were chemotactic for neutrophils and had substantial sequence homology, a C-X-C motif near the amino-terminal end, and two additional Cys residues closer to the

carboxyl-terminal end. The functions of α chemokines were mediated by receptors on the cell surface membrane (Murphy and Tiffany, 1991; Holmes *et al.*, 1991; Mueller *et al.*, 1994). Recent studies showed the presence of some types of chemokine receptors on melanoma cells. Some melanoma cells possess interleukin-8 (IL-8) receptors similar to those on neutrophils (Holmes *et al.*, 1991; Mueller *et al.*, 1994; Moser *et al.*, 1993; Norgauer *et al.*, 1996).

MGSA/GROα is a 73 amino acid peptide which shares sequence characteristics of a superfamily of peptides called α-chemokines. Richmond and colleagues initially discovered that melanoma cells secreted autostimulatory (autocrine) growth factors (Richmond *et al.*, 1983; 1985; Richmond and Thomas, 1986; Richmond *et al.*, 1982). It was found that most of the activity was caused by a single acid stable protein of about 15 kDa and designated it melanoma growth stimulatory activity (MGSA) (Richmond *et al.*, 1983; 1985). MGSA was found to be a mitogen for the melanoma cell line Hs 294T which produces this factor. MGSA was secreted by diverse melanoma cell lines but not by benign nevus cell lines (Lawson *et al.*, 1987), while immunoreactive MGSA was shown in both types of cells (Richmond *et al.*, 1986). cDNA for MGSA isolated from Hs 294T cells was later found to be identical to oncogene growth-related peptide (GROα) gene (Richmond *et al.*, 1988). The formal name for this protein was then designated as MGSA/GROα.

Recently, a second chemokine was found to be important for melanoma cell growth and metastasis in some melanoma cell lines. Schadendorf and colleagues determined that some melanoma cell lines tested secreted IL-8 (Schadendorf *et al.*, 1993). Both of two IL-8 secreting cell lines studied in more detail were dependent on IL-8 for growth. Antisense oligonucleotides targeted against human IL-8 mRNA inhibited cell proliferation, colony formation in soft agar, and secretion of IL-8 into culture supernatants. In an analysis of 13 different human melanoma cell lines, it was shown that expression of IL-8 correlates with the metastatic potential of melanoma cells in BALB/c nude mice (Singh *et al.*, 1994).

Other studies further indicate a role of these chemokines in melanoma growth and tumorigenesis. Mintz and Silvers developed a method of producing melanomas by grafting skin from Tyr-SV40E transgenic mice which are highly susceptible to melanoma to Tyr-SV40E hosts of a low susceptibility of the same inbred strain (Mintz and Silvers, 1993). It was suggested that growth factors and cytokines known to be produced in wound repair may trigger the growth and malignant conversion of melanocytes. Nanney and colleague showed that MGSA/GROα and its receptors are present in human burn wounds and may act as a mediator

for wound repair (Nanney *et al.*, 1995). MGSA/GROα and Ill-8 was induced by ultraviolet B radiation in human keratinocyte cell lines (Venner *et al.*, 1995)

IL-8 interacts with at least two distinct receptors on neutrophils (Holmes *et al.*, 1991; Murphy and Tiffany, 1991). The receptors are coupled to GTP-binding proteins, allowing transmission of the IL-8 signal into the cell (Wu *et al.*, 1993). While most of the members of the intercrine family, such as GRO and MIP2β, bind to one of the receptors, IL-8 binds to both of the IL-8 receptors (Cerretti *et al.*, 1993). The three dimensional structure of IL-8 has been elucidated by NMR (Clore *et al.*, 1990) and by X-ray crystallography (Clore and Gronenborn, 1992). A freely movable amino terminal end is followed by three beta pleated sheets and an alpha helix is located at the carboxyl-terminal end (Oppenheim *et al.*, 1991). Several lines of evidence suggest that both the amino- and carboxyl-terminal ends are involved in binding to its receptors Moser *et al.*, 1993).

Certain functions of the CXC intercrines have been elucidated by several laboratories (Yoshimura *et al.*, 1989). For example, the major functions of the IL-8 peptide appear to be related to its ability to stimulate neutrophil chemotaxis and activation (Larsen *et al.*, 1989) and to promote angiogenesis (Koch *et al.*, 1992). If neutrophils are 'primed', *e.g.*, by agents such as surface adherence or *E. coli* endotoxin (also known as lipopolysaccharide or LPS), IL-8 also stimulates the release of neutrophil enzymes such as elastase and myeloperoxidase.

High concentrations of IL-8 have also been found in inflammatory exudates in other disorders and pathological conditions in which IL-8 is thought to play an important pathogenic role (Miller and Idell, 1993). For example, IL-8 has also been implicated as a possible mediator of inflammation in rheumatoid arthritis (Seitz *et al.*, 1991) and pseudogout (Miller and Brelsford, 1993); and to have a role in cystic fibrosis (Bedard *et al.*, 1993).

Some progress has recently been made in identifying compounds capable of reducing IL-8 synthesis. Such compounds include IL-4, oxygen radical scavengers, secretory leukoprotease inhibitor and interferon gamma (Cassatella *et al.*, 1993a; 1993b), however, such studies do not concern IL-8 inhibitors. Other diverse compositions, including protein kinase C inhibitors, IL-4, and anti-IL-8 antibodies, have also been reported to modulate IL-8 actions (Mulligan *et al.*, 1993). Unfortunately, these compounds are far from ideal as candidates for use as IL-8 inhibitors in a clinical setting.

Certain progress has also been made in identifying peptide IL-8 inhibitors, however, most of such work has focused on portions of the IL-8 molecule itself (Gayle *et al.*, 1993). For

example, the present inventors have shown that synthetic peptides, and particularly, IL-8 amino terminal peptides, inhibit IL-8 binding to neutrophils and neutrophil chemotaxis (Miller *et al.*, 1993). An N-terminal pentapeptide IL-8 inhibitor has also been reported (Goodman *et al.*, 1991).

SUMMARY OF THE INVENTION

The present invention seeks to overcome the drawbacks inherent in the prior art by providing new methods for modulating and inhibiting the actions of CXC intercrine molecules such as IL-8, GRO (GRO/MGSA) and MIP2β. in controlling tumor cellproliferation. The peptides and pharmacological compositions disclosed reduce IL-8, GRO and MIP2β binding to neutrophils and inhibit IL-8-induced neutrophil activation. These peptide formulations are particularly advantageous as they are capable of inhibiting IL-8-induced enzyme release at significantly lower concentrations than is required to inhibit neutrophil chemotaxis.

Certain peptides have been found to affect melanoma cell growth. In particular, a novel hexapeptide, Antileukinate (Ac-RRWWCR-NH_2), has been found to be a potent inhibitor of binding of α-chemokines to the receptors. The effect of Antileukinate on MGSA/GROα binding to melanoma cells in several cell lines, including Hs 294T, RMPI-7951, A375P, A375SM, C8161 and WM115. suppresses the growth of the melanoma cells. Additionally, the hexapeptide also inhibits growth in lung adenocarcinoma cell lines A549, NCI-H441 and KS-LU-1 as well as in squamous cell lung cancer NCI-H292 and adenocarcinomas from stomach AGS and Hs746T, breast tissue MCF-7, prostate DU145 and colon Caco-2.

A hexapeptide, termed Antileukinate, has been identified as a potent inhibitor of binding of α-chemokines to their receptors on neutrophils. When Antileukinate was added to melanoma cells, it inhibited the binding of MGSA/GROα. The growth of cells from several melanoma cell lines was suppressed completely in the presence of 100 μM peptide. The cell growth inhibition was reversed by the removal of the peptide from the culture media or by the addition of an excess amount of MGSA/GROα. The viability of Hs 294T cells in the presence of 100 μM peptide was greater than 92%. These findings support the view that MGSA/GROα is an essential autostimulatory growth factor for melanoma cells. Antileukinate thus appears to inhibit tumor cell growth by preventing binding of the natural ligand to its receptors

The invention is generally based upon the inventors surprising discovery that relatively small peptides including the amino acid sequence Arg Arg Trp Trp Cys Xaa_1 (RRWWCX; SEQ

ID NO:23), wherein Xaa_1 is any amino acid residue, have potent anti-tumor activity. As used herein, the terms "CXC intercrine family molecules" and "CXC intercrines" are used collectively to refer to the group of peptide intercrines which include the CXC sequence motif in their N-terminal regions. CXC intercrines are known to include IL-8, GRO, MIP2α, MIP2β and ENA78, all of which molecules, and any other intercrine polypeptides that include the CXC motif, will be understood to fall within this term as used in the present application.

The inhibitory peptides of the present invention may be termed "antileukinates". Certain hexamer peptides of the sequence RRWWCX (SEQ ID NO:23) have been previously shown to have anti-bacterial activity against *Staphylococcal aureus* (Houghten *et al.*, 1991). However, there was no previously documented information to suggest that any such peptides would have an advantageous anti-tumor activity. The anti-tumor activity is particularly unexpected, particularly since the peptides are effective against a wide range of tumor cell types. It is believed that this broad activity arises because the so-called antileukin peptides herein disclosed inhibit binding of several different cytokines that affect cell growth.

In certain aspects, the present invention therefore concerns methods for inhibiting CXC intercrines, such as GRO and MIP2α or MIP2β, and particularly, IL-8. As used herein, the term "inhibiting CXC intercrines" refers to the processes by which the biological actions of the CXC intercrines are reduced. This may be particularly assessed by inhibiting their binding to one of the IL-8 receptors on their target cells, such as neutrophils, although any mode of determining CXC intercrine inhibition may be employed.

The term IL-8 is used to refer to the cytokine compositions previously known as neutrophil-activating factor, monocyte-derived neutrophil-activating peptide, monocyte-derived neutrophil-chemotactic factor and neutrophil-activating peptide-1. As used herein, the term "inhibiting IL-8" generally refers to the processes by which the biological actions of IL-8 are reduced or lessened. This includes the inhibition of any or all of the known actions of IL-8. These actions include modulating sub-cellular effects, such as receptor binding or altering cytosolic calcium levels; modulating cellular effects such as granulocyte recruitment and activation; and also affecting physiological effects, such as inflammation and angiogenesis.

In preferred embodiments, the inhibition of IL-8 function referred to in this application is the inhibition of IL-8 action on granulocytes such as neutrophils (polymorphonuclear neutrophils, PMN). This may be determined in many cellular and physiological ways, as disclosed herein. For example, by measuring inhibition of IL-8 binding to purified receptor

compositions or neutrophils; by determining the inhibition of IL-8-induced neutrophil chemotaxis or diapedesis; by measuring the inhibition of IL-8-stimulated neutrophil enzyme release (*e.g.*, myeloperoxidase, β-glucuronidase or elastase release) or superoxide production; or by assaying for anti-inflammatory effects *in vivo*, *e.g.*, using a rabbit model of dermal inflammation.

IL-8 inhibition, or indeed GRO or MIP2β inhibition, may be determined by assaying for a reduction in the intercrine binding to neutrophils, which is the most simple and straightforward method. In addition, binding of the particular intercrine to its receptor(s) must precede any other action that it has on neutrophils or other cell types. "Inhibition" of intercrines, as exemplified by the inhibition of IL-8, GRO or MIP2α or MIP2β binding to neutrophils, refers to the capacity of a given peptide or composition to inhibit intercrine binding to any detectable degree, *i.e.*, to reduce binding below the levels observed in the absence of the peptide or composition.

The inhibition of CXC intercrine binding to neutrophils may be expressed as a % Binding Inhibition value, with the higher figures representing the more effective inhibitors. The preferred peptides will generally have the higher % binding inhibition figures. Naturally, the % binding inhibition calculated will depend upon the precise assay conditions, such as the concentration of CXC intercrine and the concentration of the given peptide or composition. Conditions such as those used to generate the data of Table 1A, Table 1B, Table 2A and Table 2B, may be employed to determine whether a given peptide has any inhibitory activity. However, one may choose to employ more discriminatory conditions, such as those using lower peptide concentrations, *e.g.*, on the order of about 20 μM where one desires to obtain particularly accurate quantitative or comparative data. In any event, the determination of whether a peptide or analogue is capable of inhibiting a CXC intercrine, such as IL-8, is a straightforward matter readily achieved using assays such as those disclosed herein.

Although an understanding of the mechanism of action of the CXC intercrine inhibitors is not relevant in terms of their practical utility, it is, however, important to note that the peptide inhibitors of this invention are capable of preferentially inhibiting IL-8-induced neutrophil enzyme release at lower concentrations than IL-8-induced chemotaxis. In this sense, the term "inhibiting", when used in connection with this invention, also means "modulating" in that certain neutrophil functions are more significantly inhibited than others.

To contact a CXC intercrine or intercrine target cell with a peptide-containing composition one may simply add the peptide or composition to target cells, such as tumor cells *in vitro*. Alternatively, one may administer a biologically effective amount of a pharmacologically acceptable form of the peptide or composition to an animal, where it will contact, *e.g.*, neutrophils or macrophages and intercrines in a biological fluid *in vivo*. In this context, "contact" is achieved simply by administering the composition to the animal. Virtually any pharmaceutical peptide formulation may be used, including, but not limited to, formulations for parenteral administration, such as for intravenous, intramuscular and subcutaneous administration; inhalants, aerosols and spray formulations; formulations of peptides for topical use, such as in creams, ointments and gels; and other formulations such as peptides with lipid tails, peptides encapsulated in micelles or liposomes and drug release capsules including the active peptides incorporated within a biocompatible coating designed for slow-release.

The present invention therefore provides methods for treating cancer and other diseases and disorders associated with increased cellular proliferation.

To treat such conditions, one would identify a patient having the such a desease or proliferative disorder and then administer to the patient, preferably parenterally, a biologically effective amount of a pharmaceutical composition which includes one or more peptides of the family disclosed herein.

Naturally, one would generally tailor the particular pharmaceutical formulation according to the condition being treated. Peptides may be formulated for parenteral administration or peptides incorporated in a biocompatible coating designed for slow-release. Liposome-encapsulation may be employed, which is known to increase the efficacy and significantly prolong the half-life of administered compounds, particularly those of lower molecular weight such as the peptides disclosed herein. Various compositions and techniques for preparing all such pharmaceutical formulations will generally be known to those of skill in the art in light of the present disclosure. For a detailed listing of suitable pharmacological compositions and associated administrative techniques one may wish to refer to *Remington's Pharmaceutical Sciences*, 16th ed., 1980, Mack Publishing Co., incorporated herein by reference.

IL-8 or CXC intercrine inhibition is achieved by using a biologically effective amount of the inhibitory peptide or peptides. As used herein, a "biologically effective amount" of a peptide or composition refers to an amount effective to inhibit the actions of one or more

intercrines that affect cell growth. As disclosed herein, a variety of different peptide concentrations are very effective *in vitro*, such as those between about 100 μM and about 20 μM. Clinical doses which result in similar a local concentration of peptides are therefore contemplated to be particularly useful.

Naturally, in a clinical context, the quantity and volume of the peptide composition administered will depend on the host animal and condition to be treated and the route of administration. The precise amounts of active peptide required to be administered will depend on the judgment of the practitioner and may be peculiar to each individual. However, in light of the data presented herein, the determination of a suitable dosage range for use in humans will be straightforward. For example, doses in the order of about 0.83 mg/kg body weight/hour (mg/kg/hr) to about 16.56 mg/kg/hr, preferably about 0.83 mg/kg/hr to about 4.14 mg/kg/hr, and more preferably about 1.66 mg/kg/hr of active ingredient peptide per individual may be appropriate.

The compositions for use in inhibiting CXC intercrines, such as IL-8, GRO and MIP2α or MIPβ, in accordance with the present invention will be compositions that contain a relatively small peptide, generally of from 6 to about 14 residues in length, which includes within its sequence the amino acid sequence RRWWCX (SEQ ID NO:23). The term "a peptide" in this sense means at least one peptide, and may refer to one or more such peptides which include a sequence in compliance with the general formula RRWWCX (SEQ ID NO:23).

The relatively small peptides employed for the disclosed methods may be any length between six residues and about 14 or 15 or so residues in length, with the precise length not being an important feature of the invention. There are many advantages to using smaller peptides, for example, the cost and relative ease of large scale synthesis, and their improved pharmacological properties, such as the ease with which they can penetrate tissues and their low immunogenicity.

In addition to including an amino sequence in accordance with the sequence RRWWCX (SEQ ID NO:23), the peptides may include other short peptidyl sequences of various amino acids. The peptides may include a repeat of the sequence RRWWCX (SEQ ID NO:23) or RRWWCXX (SEQ ID NO:57). They may also contain additional sequences including, *e.g.*, short targeting sequences, tags, labeled residues, amino acids contemplated to increase the half life or stability of the peptide, or indeed, any additional residue desired for any purpose, so long

as they still function to inhibit intercrines such as IL-8 - which can be readily determined by a simple assay such as those described herein.

Amino acids which may incorporated into the peptides include all of the commonly occurring amino acids. Two designations for amino acids are used interchangeably throughout this application, as is common practice in the art: Alanine = Ala (A); Arginine = Arg (R); Aspartic Acid = Asp (D); Asparagine = Asn (N); Cysteine = Cys (C); Glutamic Acid = Glu (E); Glutamine = Gln (Q); Glycine = Gly (G); Histidine = His (H); Isoleucine = Ile (I); Leucine = Leu (L); Lysine = Lys (K); Methionine = Met (M); Phenylalanine = Phe (F); Proline = Pro (P); Serine = Ser (S); Threonine= Thr (T); Tryptophan = Trp (W); Tyrosine = Tyr (Y); Valine= Val (V).

Any of the so-called rare or modified amino acids may also be incorporated into a peptide of the invention, including the following: 2-Aminoadipic acid, 3-Aminoadipic acid, beta-Alanine (beta-Aminopropionic acid), 2-Aminobutyric acid, 4-Aminobutyric acid (piperidinic acid), 6-Aminocaproic acid, 2-Aminoheptanoic acid, 2-Aminoisobutyric acid, 3-Aminoisobutyric acid, 2-Aminopimelic acid, 2,4-Diaminobutyric acid, Desmosine, 2,2'-Diaminopimelic acid, 2,3-Diaminopropionic acid, N-Ethylglycine, N-Ethylasparagine, Hydroxylysine, allo-Hydroxylysine, 3-Hydroxyproline, 4-Hydroxyproline, Isodesmosine, allo-Isoleucine, N-Methylglycine (sarcosine), N-Methylisoleucine, N-Methylvaline, Norvaline, Norleucine and Ornithine.

The inhibitory compositions of the invention may include a peptide modified to render it biologically protected. Biologically protected peptides have certain advantages over unprotected peptides when administered to human subjects and, as disclosed in U.S. Patent 5,028,592 (incorporated herein by reference), protected peptides often exhibit increased pharmacological activity, as was found to be true in the present case.

The present invention therefore encompasses compositions comprising an acylated peptide or peptides, and preferably, a peptide acylated at the N-terminus. Although virtually any acyl group may be employed in this context, the inventors have found that the addition of an acetyl group to the N-terminus of a given peptide also renders the resultant peptide surprisingly effective at inhibiting intercrines such as IL-8. The inhibitory peptide compositions may also include a peptide(s) which is amidated at the C-terminus, *i.e.*, to which an NH_2 group has been added. In particularly preferred embodiments, peptides which have

both an acylated N-terminal and an amidated C-terminal residue are preferred as they are believed to most closely mimic natural protein and peptide structure.

Compositions for use in the present invention may also comprise peptides which include all L-amino acids, all D-amino acids or a mixture thereof. The finding that peptides composed entirely of D-amino acids have potent inhibitory activity is particularly important as such peptides are known to be resistant to proteases naturally found within the human body and are less immunogenic and can therefore be expected to have longer biological half lives.

The anti-intercrine and anti-IL-8 compositions of the present invention will generally comprise one or more peptides which include an amino acid sequence in accordance with those set forth in SEQ ID NO:1 or SEQ ID NOs:24 through 42. In certain embodiments, short hexamer peptides may be preferred. In such cases, the inhibitory compositions will generally comprise one or more peptides which have an amino acid sequence in accordance with those set forth in SEQ ID NO:1 or SEQ ID NOs:24 through 42.

The inhibitory compositions of the invention may include one or more peptides which include a sequence in accordance with the amino acid sequence Arg Arg Trp Trp Cys Arg Xaa_2 (SEQ ID NO:2). In these cases one of the variable positions has been defined as arginine and the remaining Xaa_2 may be any amino acid residue. Such sequences are exemplified by those set forth in SEQ ID NOs:3 through 22. Where short heptamer peptides are preferred, the compositions may comprise one or more peptides which have an amino acid sequence in accordance with SEQ IDs NO:3 through 22.

The invention also contemplates the use of peptides having the amino acid sequence Gln Ile Pro Arg Arg Ser Trp Cys Arg Phe Leu Phe (SEQ ID NO:52), either alone, or more preferably, in combination with one or more of the other peptides described above. The successful use of this dodecamer illustrates both the fact that longer peptides are successful and that certain biologically functional equivalent peptides are active. All such active equivalents therefore fall under the scope of the present invention.

The compositions for use in the inhibitory methods described herein may contain only a single active peptidyl species. Alternatively, they may contain more than one peptide, up to and including about 40 or 45 or so distinct peptides. Any and all of the various combinations are contemplated, such as compositions comprising 2, 3, 5, 10, 15, 20, 30 or 45 or so distinct peptides.

Compositions comprising peptides having the amino acid sequence Arg Arg Trp Trp Cys Arg (SEQ ID NO:1) and/or the amino acid sequence Arg Arg Trp Trp Cys Arg Cys (SEQ ID NO:4) are contemplated to be particularly useful in the inhibition of tumor growth and pulmonary metastasis.

The RRWWCX (SEQ ID NO:23) sequence element is an important feature of the peptides useful for practicing the invention. However, this does not exclude certain biological functional equivalents. For example, the first tryptophan in RRWWCX (SEQ ID NO:23) can be exchanged, *e.g.*, by replacing with serine, with only modest loss of activation. Therefore, one example of equivalents encompassed by the invention are peptides of the sequence RRXWCX (SEQ ID NO:58). "Equivalent amino acids" may be defined as amino acids whose hydrophilic or hydropathic index are within ± 2, more preferably, within ± 1, and most preferably, within ± 0.5 of each other. Of course, to be a "functional equivalent", a peptide must still retain its intercrine or IL-8 inhibitory activity, as may be easily determined using assays such as those disclosed herein.

In addition to the peptidyl compounds described herein, the inventors also contemplate that other sterically similar compounds, called peptidomimetics, may be formulated to mimic the key portions of the peptide structure. Such compounds may be used in the same manner as the peptides of the invention and hence are also functional equivalents. The generation of a structural functional equivalent may be achieved by the techniques of modeling and chemical design known to those of skill in the art. It will be understood that all such sterically similar constructs fall within the scope of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1A. The effect of monoclonal anti-GROα or anti-IL-8 antibody on growth of serum starved A375SM cell lines incubated with the indicated concentrations of anti-GROα mAb (closed circles), anti-IL-8 mAb (closed squares) or irrelevant isotype-specific control Ab (open circles) for 24 hours. The MTT assay was then performed (*; $p<0.03$).

FIG. 1B. The effect of monoclonal anti-GROα or anti-IL-8 antibody on growth of serum starved C8161-C cell lines incubated with the indicated concentrations of anti-GROα mAb (closed circles), anti-IL-8 mAb (closed squares) or irrelevant isotype-specific control Ab (open circles) for 24 hours. The MTT assay was then performed (*; $p<0.03$).

FIG. 2A. The effect of recombinant human GROα on growth of serum starved A375SM serum-starved cell lines incubated with the indicated concentrations of recombinant human GROα (*; $p<0.04$).

FIG. 2B. The effect of recombinant human GROα on growth of serum starved C8161-C cell lines incubated with the indicated concentrations of recombinant human GROα (*; $p<0.04$).

FIG. 3A. Inhibition of the binding of radiolabeled GROα to melanoma cell lines and melanocytes by Antileukinate. A375SM (closed circles) and C8161-C (open circles) cell lines were incubated with ^{125}I-GROα, 1 nM, in the presence of various concentrations of Antileukinate. The lines shown on the graph are the best fit curves calculated by the Lundon II computer program.

FIG. 3B. The ID_{50} of a further 12 melanoma cell lines and melanocytes are shown.

FIG. 4A. The effect of Antileukinate on growth of serum starved A375SM incubated with the indicated concentrations of Antileukinate for 24 hours (*; $p<0.035$).

FIG. 4B. The effect of Antileukinate on growth of serum starved C8161-C incubated with the indicated concentrations of Antileukinate for 24 hours (*; $p<0.035$).

FIG. 5. The effect of Antileukinate on tumor growth of A375SM in BALB/c nude mice. Human melanoma cells (1.0×10^6 cells) were injected subcutaneously and maintained for

14 days. Seven days after tumor cell injection, continuous administration of Antileukinate (closed square) or saline (open circle) was initiated (*; $p<0.043$).

FIG. 6. Production of pulmonary metastasis by human melanoma cells. Human melanoma cells (5×10^5 cells/mouse) were injected and maintained for 28 days. Lungs were then removed, fixed, and colonies of the surface were counted.

FIG. 7. Effect of Antileukinate on pulmonary metastasis. Human melanoma cells (5×10^5 cells/mouse) were injected and maintained. After 1 week post-inoculation, Antileukinate or saline (control) was administered continuously for 14 days. Four weeks post-inoculation, the lungs were removed, fixed, and colonies of the surface were counted.

FIG. 8A. The binding of ^{125}I-labeled MGSA/GROα to melanoma cell lines, Hs 294T and RPMI-7951. The melanoma cells, 1×10^5, were incubated with 1 nM ^{125}I-MGSA/GROα in the presence of various concentrations of unlabeled MGSA/GROα. Binding inhibition curve with Hs 294T (closed circles) and RPMI-7951 (open circles). The lines shown in the graph are the best fit curves calculated using the London II computer program.

FIG. 8B. The binding of ^{125}I-labeled MGSA/GROα to melanoma cell lines, Hs 294T and RPMI-7951. The melanoma cells, 1×10^5, were incubated with 1 nM ^{125}I-MGSA/GROα in the presence of various concentrations of unlabeled MGSA/GROα. Binding isotherm transformation. The total amount of labeled and unlabeled MGSA/GROα bound to the cells were calculated. Scatchard plots were shown in the inset.

FIG. 9. The specificity of the receptor binding. The melanoma cells, Hs 294T (closed symbols) and RPMI-7951 (open symbols), were incubated with indicated concentrations of radiolabeled MGSA/GROα (circles) or IL-8 (triangles). The non-specific binding was estimated by the binding in the presence of 100 fold excess of unlabeled material. Specific binding which is calculated by subtracting non-specific binding from total binding is shown in this figure. IL-8 did not bind specifically to the melanoma cells.

FIG. 10. The effect of Antileukinate on the binding of radiolabeled MGSA/GROα to the melanoma cell lines. The melanoma cells, Hs 294T (closed circles) and RPMI-7951 (open circles), were incubated with 1 nM ^{125}I-MGSA/GROα in the presence of different concentrations of Antileukinate. The lines shown in the graph are the best fit curves calculated using the London II computer program.

FIG. 11A. Analysis for the breakdown of Antileukinate in the culture of Hs 294T cells. A total concentration of tritium labeled and unlabeled Antileukinate of 100 μM, was added to the culture of Hs 294T cells. Aliquots of medium were removed at different times and chromatographed on an HPLC Bondapak C18 reversed phase column. The peptides were eluted using a gradient from 0.1% TFA to 80% acetonitrile in 0.1% TFA. One milliliter fractions were collected and measured for their radioactivity using a liquid scintillation counter. The chromatographs show the elution volume of the radioactivity at different times after addition to the cells. The peptide is substantially eliminated from the culture within 24 hr.

FIG. 11B. Analysis for the breakdown of Antileukinate in the culture of Hep G2 cells. A total concentration of tritium labeled and unlabeled Antileukinate of 100 μM, was added to the culture of Hep G2 cells. Aliquots of medium were removed at different times and chromatographed on an HPLC Bondapak C18 reversed phase column. The peptides were eluted using a gradient from 0.1% TFA to 80% acetonitrile in 0.1% TFA. One milliliter fractions were collected and measured for their radioactivity using a liquid scintillation counter. The chromatographs show the elution volume of the radioactivity at different times after addition to the cells. The peptide is substantially eliminated from the culture within 24 hr.

FIG. 12A. The effect of Antileukinate on the growth of melanoma cells. Melanoma cell lines and a control liver cancer cell line (2×10^4 cells/well) in 500 μl were cultured in a 24 well tissue culture plate in the presence of various concentrations of Antileukinate for 3 d. The culture media were changed to fresh media containing the same concentration of Antileukinate every 24 hr during the incubation period. After incubation, the cells were collected and the cell number was counted using a hemocytometer. Hs 294 T cell line. Analysis of variance was used for multiple comparison. When there was significant difference, the differences between the

number of cell without the peptide and those with peptide were tested using The Sheffe's Test; *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$.

FIG. 12B. The effect of Antileukinate on the growth of melanoma cells. Melanoma cell lines and a control liver cancer cell line (2×10^4 cells/well) in 500 µl were cultured in a 24 well tissue culture plate in the presence of various concentrations of Antileukinate for 3 d. The culture media were changed to fresh media containing the same concentration of Antileukinate every 24 hr during the incubation period. After incubation, the cells were collected and the cell number was counted using a hemocytometer. RPMI-7951 cell line. Analysis of variance was used for multiple comparison. When there was significant difference, the differences between the number of cell without the peptide and those with peptide were tested using The Sheffe's Test; *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$.

FIG. 12C. The effect of Antileukinate on the growth of melanoma cells. Melanoma cell lines and a control liver cancer cell line (2×10^4 cells/well) in 500 µl were cultured in a 24 well tissue culture plate in the presence of various concentrations of Antileukinate for 3 d. The culture media were changed to fresh media containing the same concentration of Antileukinate every 24 hr during the incubation period. After incubation, the cells were collected and the cell number was counted using a hemocytometer. Liver cancer cell line, Hep G2. Analysis of variance was used for multiple comparison. When there was significant difference, the differences between the number of cell without the peptide and those with peptide were tested using The Sheffe's Test; *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$.

FIG. 13A. Restoration of Hs 294T cell growth inhibition by removal of Antileukinate or the addition of excess MGSA/GROα. Melanoma cell lines Hs 294T (2×10^4 cells/well) were cultured in a 24 well tissue culture plate for 1 to 8 d, and the cell number was counted using a hemocytometer. The effect of removal of Antileukinate from the cell culture. The cell culture was started in the presence of 50 µM Antileukiante. 24 hr later, the culture media was replaced using media with Antileukinate and the culture was continued in the absence of the peptide (open triangles). The cell numbers were compared with those measured when the cells were cultured in the presence (open squares) or the absence (open circles) of the peptide throughout the culture periods. When there was significant difference, the Scheffe's Test

was performed to establish the significance between two groups. The data significantly different from those with Antileukinate (+) were marked; *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$.

FIG. 13B. Restoration of Hs 294T cell growth inhibition by removal of Antileukinate or the addition of excess MGSA/GROα. Melanoma cell lines Hs 294T (2×10^4 cells/well) were cultured in a 24 well tissue culture plate for 1 to 8 d, and the cell number was counted using a hemocytometer. The effect of addition of excess MGSA/GROα from the cell culture. The cell culture was started in the presence of 50μM Antileukinate. 24 hr later, the culture media was replaced using media containing Antileukinate and 50 nM MGSA/GROα (closed squares). The cell numbers were compared with those measured when the cells were cultured in the presence of Antileukinate alone (open squares). Analysis of variance was used for multiple comparisons of the cell numbers obtained at each culture period. When there was significant difference, the Scheffe's Test was performed to establish the significance between two groups. The data significantly different from those with Antileukinate (+) were marked; *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$.

FIG. 14. The viability of Hs 294T cells after the culture for 72 hr in the presence of Antileukinate. Melanoma cell line Hs 294T was cultured in the presence of various concentrations of Antileukinate for 72 hr. The viability of the cells was measured by means of trypan blue dye exclusion. Median ± 25% percentile range of the data are shown.

FIG. 15. The effect of Antileukinate on growth of DU145 (adenocarcinoma of prostate) cell line. Serum-starved DU145 were incubated with the indicated concentrations of Antileukinate for 24 hours. MTT assay was then performed. Cell number was decreased in a dose-dependent manner and there are significant differences from controls at concentrations greater than, or equal to 100 μM of Antileukinate ($p<0.05$).

FIG. 16. The effect of Antileukinate on growth of NCI-H292 (squamous cell carcinoma of lung). NCI-H292 cells were plated at a density of $5x10^3$ cells in 24-well plate with growth medium. After the cells adhered to the plate, supernatants were aspirated and culture media containing various concentrations of Antileukinate were added to the wells. The media was replaced every 24 hour and the cells were cultured for 7 days. After incubation, cells

were detatched with trypsin-EDTA and the cell number was counted using a hemocytometer. Cell number was decreased in a dose-dependent manner and there are significant differences from control at concentrations greater than, or equal to 50 μM of Antileukinate ($p<0.05$).

FIG. 17 The effect of Antileukinate on growth of A549 (adenocarcinoma of lung). A549 cells were plated at a density of $5x10^3$ cells in 24-well plate with growth medium. After the cells adhered to the plate, supernatants were aspirated and culture media containing various concentrations of Antileukinate were added to the wells. The media was replaced every 24 hours and the cells were cultured for 7 days. After incubation, cells were detatched with trypsin-EDTA and the cell number was counted using a hemocytometer. Cell number was decreased in a dose-dependent manner and there are significant differences from control at concentrations greater than, or equal to 50 μM of Antileukinate ($p<0.05$).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Growth-related oncogene (GROα) was first described as an autocrine mitogen and growth factor for melanoma cells. More recent studies show that GROα, IL-8 and other members of the α-chemokine superfamily, are also angiogenic. Melanocytes and twelve human melanoma cell lines produce both GROα and IL-8. The proliferation of A375SM, a highly metastatic cell line, and C8161-C were significantly increased by *r*GROα and inhibited by anti-human GROα mAb. Antileukinate, a potent inhibitor of α-chemokine receptor binding, inhibited the binding of GROα to its receptors on melanocytes and all twelve melanoma cell lines tested. Antileukinate also suppressed proliferation of A375SM and C8161-C cells in a dose-dependent manner, and the suppression was not due to cytotoxic effects. Continuous administration of Antileukinate inhibited the tumor growth and pulmonary metastasis of A375SM cells in athymic BALB/c nude mice. Antileukinate may inhibit the growth of melanoma cells by preventing GROα from binding to its receptors, indicating use of α-chemokine receptor inhibitors, such as Antileukinate in the treatment of malignant melanoma.

Malignant melanoma is the seventh leading cancer in the United States and the incidence for melanoma is increasing faster than that of any other cancers . Despite extensive research, for patients with inoperable tumors, the prognosis remains extremely poor.

Growth-related oncogene GROα is a 73 amino acid polypeptide of the α-chemokine superfamily of proteins. Originally, GROα was described as an autocrine growth factor produced by melanoma cells (13). However, further studies have shown that GROα is produced by a variety of cell types including melanoma cells, monocytes, keratinocytes, and lung cancer cells (12). IL-8, another α-chemokine of approximately 72 amino acid residues has been described as a neutrophil chemotactic and activating factor, and has been shown to play an important role in angiogenesis. The functions of these α-chemokines are mediated by specific receptors on the cell surface membrane. Neutralizing antibodies for IL-8 or GROα inhibit their functions of angiogenesis and growth stimulation of melanoma cell lines (14).

Antileukinate (Ac-RRWWCR-NH_2) is a potent inhibitor of binding of α-chemokines to the receptors and it inhibits the proliferation of melanoma cells *in vitro* (13). GROα, which acts as a specific autocrine growth factor for the cells, is produced by melanocytes and multiple human melanoma cell lines, and Antileukinate inhibits tumor growth and pulmonary metastasis of the human melanoma cells *in vivo*.

CXC Intercrines, IL-8 Actions and Inhibitory Peptides

Studies have also been conducted on the interaction of IL-8 with its receptors. This has led to the identification of certain peptide inhibitors with structures corresponding to portions of the IL-8 molecule. For example, Gayle and colleagues found that the 44 amino acids at the amino-terminal end of the rabbit IL-8 receptor was a moderately good inhibitor of IL-8 binding and function (Gayle *et al.*, 1993). Synthetic peptides, and particularly, IL-8 amino terminal peptides, inhibit IL-8 binding to neutrophils and neutrophil chemotaxis. An N-terminal pentapeptide IL-8 inhibitor has also been reported (Goodman *et al.*, 1991).

Various other peptide compositions for IL-8 inhibitory activity have been assayed by screening a library of 400 groups of hexapeptides. In these screening assays, ^{125}I-labeled interleukin-8 (10^{-12} M) was mixed with 100 μM peptide, then added to neutrophils and incubated at 4°C for 90 min. The bound radioactivity was separated from unbound by centrifugation through a dense cushion of a mixture of paraffin and silicon oils and the ^{125}I bound to neutrophils was counted in a gamma radiation spectrometer, allowing the results to be expressed as the percent of IL-8 binding which was inhibited.

Hexamers of the sequence RRWWCX (SEQ ID NO:23), where the terminal position may be any amino acid, were found to be effective IL-8 inhibitors. RRWWCR effectively inhibits other CXC intercrines, such as GRO and MIP2, as evidenced by reducing GRO and MIP2β binding to human neutrophils.

Although all RRWWCX (SEQ ID NO:23) series peptides have anti-IL-8 activity (Table 1A and Table 1B), the peptide RRWWCR (SEQ ID NO:1) was found to be particularly effective. A form of this peptide with an amino-terminus acetyl group and a carboxy-terminus amino group (Ac-RRWWCR-NH_2; SEQ ID NO:1), thus modified in order to resemble peptides present among longer sequence of protein, became one of the focal points of these studies.

Ac-RRWWCR-NH_2 (SEQ ID NO:1) was found to inhibit the specific binding of ^{125}I-labeled IL-8 to neutrophils with an apparent K_I of approximately 10 μM, and to be almost twice as effective as the non-acetylated form of the same peptide. A precise K_I value could not be obtained due to the presence of positive cooperativity. The binding isotherm of IL-8 in the absence of the peptide fitted one-site model best, when it was analyzed using the computer program Lundon I. One possible explanation of the lower IL-8 concentration data in the Scatchard plots is that cooperativity masked the high affinity binding at low IL-8 concentrations. Recent studies, however, have shown that IL-8 bound to two distinct classes of IL-8 receptors with almost similar affinity (Lee *et al.*, 1982; Schumacher *et al.*, 1992). Therefore, it is more likely that the estimated Bmax for this binding site as one-site model represents the total Bmax of two class of receptors and that the estimated Kd is common for these receptors.

The binding isotherms in the presence of the peptide fit two-site model best. The analysis of binding isotherms in the presence of Ac-RRWWCR-NH_2 showed that this peptide suppressed the binding of IL-8 to two classes of receptors differently. The estimated values of binding parameters showed that affinity of one class of receptors was suppressed by 10 μM peptide, which suggested competitive inhibition. Higher concentration of peptide is needed to inhibit the other class of receptor non-competitively.

The activity of the present inhibitory peptides is specific for IL-8. Ac-RRWWCR-NH_2 does not inhibit the binding of C5a or leukotriene B_4 to neutrophils, chemotaxis induced by formyl-L-Met-L-Leu-L-Phe (fMLP), or β-glucuronidase release induced by fMLP, C5a or leukotriene B_4. It also has no intrinsic ability to cause neutrophil chemotaxis or enzyme release.

Ac-RRWWCR-NH_2 (Antileukinate) is a potent inhibitor of binding of α-chemokines to the receptors on the neutrophils. Antileukinate inhibited the binding of radiolabeled IL-8 with an IC_{50} of 13.7 μM. The activity of the peptide was specific for α-chemokines. Antileukinate also suppressed the binding of radiolabeled macrophage inflammatory peptide-2β (MIP-2β) to human neutrophils, while it did not affect the binding of radiolabeled MIP-1α, leukotriene B_4 or C5a. Antileukinate inhibited the enzyme release from neutrophils stimulated by IL-8 with an EC_{50} of 0.8 μM. The peptide binds to human neutrophils, while it does not interact with IL-8. Together with the finding that some part of IL-8 binding to human neutrophil is competitively inhibited by Antileukinate, these findings suggest that Antileukinate interacts with α-chemokine receptors rather than with the ligand.

A second set of peptides which contained Ac-rrwwcrx-NH_2 (SEQ ID NO:2), with all D-amino acids showed inhibitory activity Again, all RRWWCRX (SEQ ID NO:2) series peptides were found to have anti-IL-8 activity (Table 5A and Table 2B). However, the peptide Ac-rrwwcrc-NH_2 (SEQ ID NO:4) was found to be the best inhibitor, being almost four times as potent an inhibitor as Ac-rrwwcr-NH_2 (SEQ ID NO:1). This observation is potentially of great significance as mammalian proteases cannot degrade D-amino acid peptides and proteins (Togo *et al.*, 1989). Therefore, D-amino acid peptide inhibitors are expected to have a longer half life *in vivo*.

Various other synthetic peptides were also tested for their ability to inhibit IL-8 binding to neutrophils in the standard assay. These peptides were either homologues of the amino-terminal end of IL-8, or were segments of proteins found in the protein data bases (PIR or Swiss-Prot) which had five of the six residues in RRWWCR (SEQ ID NO:2). The latter peptides were identified by searching the PIR and Swiss-Prot databases for RRWWCR (SEQ ID NO:1), using the IGSUITE program to search the databases present on the CRAY computer at the Center for High Performance Computing in Austin Texas. None of the proteins in the data bases contained all six of the amino acids.

Previous studies have shown that the residues Glu, Leu and Arg at the 4, 5, and 6 positions of the 72 amino acid of IL-8 are important for the binding to neutrophils (Clore *et al.*, 1992), and that the amino terminal peptide of IL-8 inhibits IL-8 binding to neutrophils and chemotaxis (Miller *et al.*, 1993). The IL-8 homologues Ac-KELRCQ-NH_2 (SEQ ID NO:54) and ELRCQCIKTY (SEQ ID NO:49 were examined, including the C-X-C motif characteristic of intercrine peptides), along with its two non-Cys-containing analogues, ELRSQSKTY (SEQ

ID NO:50) and ELRMQMKTY (SEQ ID NO:51). None of these peptides had the ability to inhibit IL-8 binding to neutrophils.

QIPRRSWCRFLF (SEQ ID NO:52), inhibited IL-8 binding to neutrophils by about 60%, which was less than Ac-RRWWCR-NH_2 (SEQ ID NO:1). This suggested that the first tryptophan in RRWWCR (SEQ ID NO:1) can be modified with only modest loss of activation. The lack of activity of the other peptide, GRRWWCDAVLY (SEQ ID NO:53), suggests that changing the last Arg in RRWWCR (SEQ ID NO:1) to Asp significantly reduces its ability to inhibit IL-8 binding to neutrophils, as supported by the observation that Ac-rrwwcd (SEQ ID NO:26) is only minimally active.

The peptide inhibitors of the RRWWCX (SEQ ID NO:23) and RRWWCRX (SEQ ID NO:2) types have the distinct advantage over previously described inhibitors in that they are only six or seven residues long and that the D-amino acid analogues are also active.

Biological Functional Equivalents

Certain biological functional equivalents of the RRWWCXX-type peptides are contemplated within the scope of this invention. The concept of biologically functional equivalent amino acids is well known to those of skill in the art, and is embodied in the knowledge that modifications and changes may be made in the structure of a protein or peptide and still obtain a molecule having like or otherwise desirable characteristics.

However, it is also well understood by skilled artisans that, inherent in the definition of a biologically functional equivalent protein or peptide, is the concept that there is a limit to the number of changes that may be made within a defined portion of the molecule and still result in a molecule with an acceptable level of equivalent biological activity and that key active site or structurally vital residues cannot be exchanged. Biologically functional equivalent peptides are therefore defined herein as those peptides in which certain, not most or all, of the amino acids may be substituted. In particular, where hexamer or heptamer peptides are concerned, it is contemplated that only about two, or more preferably, a single amino acid change would be made within a given peptide. Of course, a plurality of distinct peptides with different substitutions may easily be made and used in accordance with the invention.

In regard to changing a limited number of residues within a peptide, it is known that certain amino acids may be substituted for other amino acids without appreciable loss of function, as may be measured by the interactive binding capacity for structures such as

receptors and cells, or the ability to compete with other molecules for binding to these sites. Since it is the interactive and competitive capacity of a protein or peptide that defines its biological functional activity, certain amino acid substitutions can be made in a peptide sequence (or, of course, its underlying DNA coding sequence) and nevertheless obtain a peptide with like, or even improved properties.

Amino acid substitutions are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. An analysis of the size, shape and type of the amino acid side-chain substituents reveals that arginine, lysine and histidine are all positively charged residues; that alanine, glycine and serine are all a similar size; and that phenylalanine, tryptophan and tyrosine all have a generally similar shape. Therefore, based upon these considerations, arginine, lysine and histidine; alanine, glycine and serine; and phenylalanine, tryptophan and tyrosine; are defined herein as biologically functional equivalents.

To effect more quantitative changes, the hydropathic index of amino acids may be considered. Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics, these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (glutamic acid) (-3.5); glutamine (-3.5); aspartate (aspartic acid) (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is generally understood in the art (Kyte and Doolittle, 1982, incorporated herein by reference). It is known that certain amino acids may be substituted for other amino acids having a similar hydropathic index or score and still retain a similar biological activity. In making changes based upon the hydropathic index, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

Substitution of like amino acids can also be made on the basis of hydrophilicity, as disclosed in U.S. Patent 4,554,101, incorporated herein by reference. In U.S. Patent 4,554,101, the following hydrophilicity values are assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (aspartic acid) (+3.0 ± 1); glutamate (glutamic acid) (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); proline (-0.5 ± 1); threonine (-0.4); alanine

(-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

It is well understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically functional equivalent protein or peptide. In making changes based upon similar hydrophilicity values, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

Pharmaceutical Formulations

The peptides and compositions of the invention may be used for treating a variety of diseases and disorders in which CXC intercrines, such as IL-8, or neutrophils are involved or in which there is an inappropriate or increased inflammatory response.

As the invention may be employed to treat tumor cell proliferation and metastasis in various clinical settings, many types of pharmaceutical peptide formulations are contemplated. Therapeutic or pharmacological compositions of the present invention, whether for pulmonary or other treatments, will generally comprise an effective amount of a relatively small intercrine- or IL-8-inhibiting peptide or peptides, dissolved or dispersed in a pharmaceutically acceptable medium. The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an allergic, toxic, or otherwise adverse reaction when administered to a human. Pharmaceutically acceptable media or carriers include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated.

Supplementary active ingredients can also be incorporated into the therapeutic compositions of the present invention. For example, the intercrine-, IL-8- and neutrophil-inhibiting peptides may also be combined with other agents such as IL-8-derived N-terminal peptides, IFN-γ, oxygen radical scavengers and the like, to create peptide cocktails for treatment.

The preparation of pharmaceutical or pharmacological compositions containing an intercrine-, IL-8- and neutrophil-inhibiting peptide or peptides, including dextrorotatory peptides, as an active ingredients will be known to those of skill in the art in light of the present

disclosure. If desired, such compositions may be prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection; as tablets or other solids for oral administration; as time release capsules; or in any other form currently used.

Solutions of the active peptides and compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

Sterile solutions suitable for injection are contemplated to be useful in treating various diseases and may be administered into the blood stream or into the precise site of the inflammation. The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

A peptide or peptides can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine, and the like.

The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial ad antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride.

Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The preparation of more, or highly, concentrated solutions for intramuscular injection is also contemplated. In this regard, the use of DMSO as solvent is preferred as this will result in extremely rapid penetration, delivering high concentrations of the active peptide, peptides or agents to a small area.

Upon formulation, therapeutics will be administered in a manner compatible with the dosage formulation, and in such amount as is pharmacologically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed.

A minimal volume of a composition required to disperse the peptide is typically utilized. Suitable regimes for administration are also variable, but would be typified by initially administering the compound and monitoring the results and then giving further controlled doses at further intervals. For example, for parenteral administration, a suitably buffered, and if necessary, isotonic aqueous solution would be prepared and used for intravenous, intramuscular, subcutaneous or even intraperitoneal administration. One dosage could be dissolved in 1 mL of isotonic NaCl solution and either added to 1000 mL of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580).

In certain embodiments, active compounds may be administered orally. This is contemplated for agents which are generally resistant, or have been rendered resistant, to proteolysis by digestive enzymes. Such compounds are contemplated to include dextrorotatory

peptides; chemically designed or modified agents; and peptide and liposomal formulations in time release capsules to avoid peptidase and lipase degradation.

Oral formulations may include compounds in combination with an inert diluent or an assimilable edible carrier; those enclosed in hard or soft shell gelatin capsules; those compressed into tablets; or those incorporated directly with the food of the diet. For oral therapeutic administration, the active compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tables, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should generally contain at least 0.1% of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 60% of the weight of the unit. The amount of active compounds in such therapeutically useful compositions is such that a suitable dosage will be obtained.

Tablets, troches, pills, capsules and the like may also contain the following: a binder, as gum tragacanth, acacia, cornstarch, or gelatin; excipients, such as dicalcium phosphate; a disintegrating agent, such as corn starch, potato starch, alginic acid and the like; a lubricant, such as magnesium stearate; and a sweetening agent, such as sucrose, lactose or saccharin may be added or a flavoring agent, such as peppermint, oil of wintergreen, or cherry flavoring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup of elixir may contain the active compounds sucrose as a sweetening agent methyl and propylparabens as preservatives, a dye and flavoring, such as cherry or orange flavor. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compounds may be incorporated into sustained-release preparation and formulations.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE I

HEXAMER PEPTIDE INHIBITORS OF IL-8

A series of studies was first carried out to determine whether hexamer peptides of the sequence RRWWCX (SEQ ID NO:23), where X may be any amino acid, would act as inhibitors of IL-8. The assays of the initial screen are based upon determining the ability of a given peptide to inhibit the binding of IL-8 to human neutrophils.

A. Preparation of Human Neutrophils

The use of human subjects for acquisition of neutrophils was approved by the Institutional Review Board for human experimentation. For the preparation of neutrophils, human blood was anticoagulated with heparin for enzyme release studies and with 0.33% sodium citrate for other studies. For chemotactic and enzyme release studies neutrophils were separated by dextran sedimentation and erythrocyte lysis by the method of Boyum (Kohler and Milstein, 1975). For binding studies and cytotoxic studies, the neutrophils were further purified in gradients of Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) to a purity of 90-93% (Kohler and Milstein, 1975).

B. IL-8 Binding Assays

Recombinant human IL-8 (72 amino acids; Pepro Tech Inc., Rocky Hill, NJ) was radioactively labeled with ^{125}I by the chloramine T method of Hunter and Greenwood (Hunter and Greenwood, 1962). Binding studies were performed according to Besemer *et al.* (1989), as follows: Neutrophils in phosphate buffered saline pH 7.4 (PBS) containing 0.1% bovine serum albumin (BSA) were incubated with labeled ligand in the presence or absence of the peptide being tested for 90 min at 4°C to reach equilibrium and then centrifuged at 12,000 × g for 3 min in Beckman B microfuge (Beckman Instruments, Fullerton, CA), through a cushion consisting of a mixture of paraffin oil (Fisher Scientific, Fair Lawn, NJ) and silicon oil (Serva Co., N.Y., NY). The pellet and supernatant were then counted in a gamma radiation spectrometer. The non-specific binding was estimated to measure the binding in the presence of 100-fold excess of non-labeled ligand. The binding constants were calculated using the computer programs Lundon I and Lundon II.

C. Peptides

The RRWWCR-type peptides were synthesized by Houghten Pharmaceutical Company in San Diego using tBOC for protection of the α-amino group (Stewart and Young, 1969). All synthetic peptides were purified on high performance liquid chromatography (HPLC) using a preparative C18 reverse phase column (Waters Co., New Bedford, MA). Peptides were eluted using a gradient from 0.1% trifluoroacetic acid (TFA) to 80% acetonitrile in 0.1% TFA. The composition of the peptides was confirmed by amino acid analysis and sequencing.

D. Statistics

In this and all of the following examples, the data are expressed as the mean and the variation as the standard deviation (S.D.). Significance was determined by the Sheffés test when the variances were equal and the populations were normally distributed and only 2 groups were compared. Multiple comparisons were made using the analysis of variance and Sheffe's test.

E. Results

In screening for inhibition was carried out at 100 μM concentration of the peptide being tested, with a 10^{-12} M concentration of IL-8. In the first series of studies, twenty peptides were synthesized with the carboxyl-terminal residue of RRWWCX (SEQ ID NO:23) being changed to each of the standard protein amino acids in turn. In the first set of studies, several of the peptides totally inhibited IL-8 binding, as shown in Table 1A and Table 1B. The information presented in Table 1A and Table 1B is the same data, with Table 1A being listed in order of % inhibition and Table 1B being listed in order of SEQ ID NO, to enable straightforward comparisons. It can be clearly seen that all hexamer peptides other than RRWWCD (SEQ ID NO:26); RRWWCE (SEQ ID NO:27); RRWWCN (SEQ ID NO:35) and RRWWCQ (SEQ ID NO:37), have very significant inhibitory activity. It should also be noted that even though the preliminary data in Table 1 shows RRWWCN (SEQ ID NO:35) not to have inhibitory activity, subsequent studies showed this peptide did indeed exhibit certain inhibitory properties (FIG. 1).

TABLE 1A

PEPTIDE	SEQ ID NO:	% INHIB.
RRWWCR	1	112.2
RRWWCK	32	110.3
RRWWCT	39	88.4
RRWWCP	36	88.3
RRWWCH	30	87.9
RRWWCL	33	86.9
RRWWCG	29	86.8
RRWWCV	40	81.1
RRWWCF	28	80.2
RRWWCS	38	79.3
RRWWCY	42	77.9
RRWWCC	25	75.1
RRWWCM	34	69.0
RRWWCW	41	66.4
RRWWCA	24	51.9
RRWWCI	31	45.8
RRWWCQ	37	19.2
RRWWCE	27	17.1
RRWWCD	26	11.8
RRWWCN	35	-12.0

TABLE 1B

PEPTIDE	SEQ ID NO:	% INHIB.
RRWWCR	1	112.2
RRWWCA	24	51.9
RRWWCC	25	75.1
RRWWCD	26	11.8
RRWWCE	27	17.1
RRWWCF	28	80.2
RRWWCG	29	86.8
RRWWCH	30	87.9
RRWWCI	31	45.8
RRWWCK	32	110.3
RRWWCL	33	86.9
RRWWCM	34	69.0
RRWWCN	35	-12.0
RRWWCP	36	88.3
RRWWCQ	37	19.2
RRWWCS	38	79.3
RRWWCT	39	88.4
RRWWCV	40	81.1
RRWWCW	41	66.4
RRWWCY	42	77.9

As shown in Table 1A and Table 1B, several of the peptides totally inhibited IL-8 binding in the first studies. To assess relative effectiveness, the group was therefore re-evaluated with lower concentrations of the peptides. In these studies, Ac-RRWWCR-NH_2 (SEQ ID NO:1) was found to be the most potent inhibitor of binding of IL-8 to neutrophils (FIG. 1). The data presented show RRWWCR (SEQ ID NO:1) to be significantly better than the other peptides under these conditions.

In another initial study, the acetylated derivative of RRWWCR (SEQ ID NO:1; Ac-RRWWCR-NH_2) emerged to be almost twice as effective as the non-acetylated peptide in inhibiting IL-8 binding to neutrophils.

EXAMPLE II

SPECIFICITY OF RRWWCR TO IL-8 BINDING INHIBITION

To examine the specificity of Ac-RRWWCR-NH_2 (SEQ ID NO:1) on ligand-receptor binding, the inventors tested whether the peptide reduced the binding of human ^{125}I-C5a or ^{3}H-leukotriene B_4 to human neutrophils.

Recombinant human fifth component of complement (C5a) (Sigma Chemical Co., St. Louis, MO) was radioiodinated enzymatically using Enzymobead (Bio Rad, Richmond, CA). Tritiated leukotriene B_4 was purchased from Du Pont Co., Wilmington, DE. Binding assays for C5a and leukotriene B_4 were performed as described for IL-8 binding in Example I, except that the buffers used were PBS containing 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.5% BSA, and HBSS containing 0.1% ovulabumin and 10 mM HEPES (pH7.3), respectively, as previously described (Braunwalder *et al.*, 1992; Sherman *et al.*, 1988). The radioactivity was measured with liquid scintillation counter for the leukotriene B_4 assay. The binding of both ligands were saturable and inhibited by the non-labeled agonists in dose dependent manners.

In these studies it was found that at 100 μM Ac-RRWWCR-NH_2 (SEQ ID NO:1) did not affect the binding of C5a nor leukotriene B_4, but that it suppressed the IL-8 binding significantly at a concentration of 100 nM .

EXAMPLE III

CYTOTOXICITY OF RRWWCR TO NEUTROPHILS

Cytotoxic capacity of Ac-RRWWCR-NH_2 (SEQ ID NO:1 was determined by measuring the amount of ^{51}Cr released from neutrophils, as follows: The neutrophil preparation (2 ×

10^7/ml) in RPMI-1640 media containing 10% donor's plasma was incubated with 500 μCi of $Na_2{}^{51}CrO_4$ (Du Pont Co.) for 60 min at 37°C. The cells were washed 3 times, resuspended in the media at the concentration of 1×10^7/ml, and then incubated for 30 min at 37°C to allow spontaneous lysis of marginally viable cells. After washing twice, a 100 μl aliquot of the ^{51}Cr-labeled neutrophils (5×10^6/ml) was mixed with 100 μl of Ac-RRWWCR-NH_2 (SEQ ID NO:1) in a siliconized microcentrifuge tube. The buffer and the conditions of incubation simulated either the binding assay or the chemotactic assay. After the incubation, the tubes were centrifuged at 300×g for 7 min at 4°C and the radioactivity in the supernatant was then counted in a gamma radiation spectrometer. Triplicate tubes containing buffer alone or 2% SDS were used to determine spontaneous and maximum release, respectively. The percentage lysis was calculated by using the following formula:

$$\%\ \text{LYSIS} = \frac{(\text{EXPERIMENTAL CPM} - \text{SPONTANEOUS CPM})\ X100}{(\text{MAXIMUM CPM} - \text{SPONTANEOUS CPM})}$$

When chromium-labeled cells were incubated in PBS containing 0.1% BSA for 90 min at 4°C, it was found that the percentage of cells lysed remained near control level up to 500 μM of the peptide. Under conditions used in chemotaxis, 100 μM of the peptide had no effect, however, 500 μM peptide damaged almost 25% of the cells.

EXAMPLE IV

ADDITIONAL PEPTIDE INHIBITORS OF IL-8

A. Heptamer Peptides

D-amino acid analogues of RRWWCR (SEQ ID NO:1) with an added seventh amino acid. In the heptamer studies, twenty peptides were synthesized with the carboxyl-terminal residue of RRWWCRX (SEQ ID NO:2) being changed to each of the standard protein amino acids in turn. In the first set of heptamer studies, several of the peptides exhibited very strong inhibition of IL-8 binding, as shown in Table 2A and Table 2B. The information presented in Table 2A and Table 2B is the same data, with Table 2A being listed in order of % inhibition and Table 2B being listed in order of SEQ ID NO, to enable straightforward comparisons. It can be clearly seen that all heptamer peptides (SEQ ID NOs:3 through 22) have significant inhibitory activity under these conditions (Table 5A).

TABLE 2A

PEPTIDE	SEQ ID NO:	% INHIB.
RRWWCR	1	112.2
rrwwcrk	11	113.1
rrwwcrn	14	110.8
rrwwcrg	8	108.0
rrwwcrc	4	107.7
rrwwcrd	5	107.0
rrwwcrh	9	107.0
rrwwcrw	21	106.3
rrwwcrf	7	105.6
rrwwcrr	17	102.8
rrwwcre	6	99.1
rrwwcrl	12	99.1
rrwwcrm	13	96.2
rrwwcra	3	95.3
rrwwcri	10	93.9
rrwwcrq	16	89.2
rrwwcrv	20	89.2
rrwwcrp	15	87.3
rrwwcry	22	85.4
rrwwcrs	18	70.4
rrwwcrt	19	56.3

TABLE 2B

PEPTIDE	SEQ ID NO:	% INHIB.
RRWWCR	1	112.2
rrwwcra	3	95.3
rrwwcrc	4	107.7
rrwwcrd	5	107.0
rrwwcre	6	99.1
rrwwcrf	7	105.6
rrwwcrg	8	108.0
rrwwcrh	9	107.0
rrwwcri	10	93.9
rrwwcrk	11	113.1
rrwwcrl	12	99.1
rrwwcrm	13	96.2
rrwwcrn	14	110.8
rrwwcrp	15	87.3
rrwwcrq	16	89.2
rrwwcrr	17	102.8
rrwwcrs	18	70.4
rrwwcrt	19	56.3
rrwwcrv	20	89.2
rrwwcrw	21	106.3
rrwwcry	22	85.4

In order to determine the relative effectiveness of the heptamers, their inhibitory effects were determined at lower concentrations. The peptide with D-cysteine present at the carboxyl-terminal end (RRWWCRC; SEQ ID NO:4) was found to be almost 56% more effective than the next best peptide in this group and to be more effective than Ac-RRWWCR-NH_2 (SEQ ID NO:1). Ac-rrwwcrc-NH_2 (SEQ ID NO:4) prevented 80% of the binding of IL-8 to neutrophils as compared to 20% inhibition by the L-amino acid peptide Ac-RRWWCR-NH_2 (SEQ ID NO:1) at 10 μM.

B. Other Peptides

Additional peptides which were either related to the amino terminal portion of IL-8 or were found in other proteins and had five of the six residues in RRWWCR (SEQ ID NO:1) were tested The peptides ELRCQCIKTY, ELRSQSIKTY, ELRMQMIKTY, QIPRRSWCRFLF, and GWRRWWCDAVLY (SEQ ID NOs:49 through 53, respectively) were synthesized at The University of Texas Health Center at Tyler utilizing an 431 Peptide Synthesizer (Applied Biosystems, Foster City, CA), using the 9-fluorenylmethoxycarbonyl (fMOC) group to protect the α-amino group as described by Meienhofer and coworkers (Meienhofer, *et al.*, 1979; Arshady, *et al.*, 1979). All synthetic peptides were purified on high performance liquid chromatography (HPLC) using a preparative C18 reverse phase column (Waters Co., New Bedford, MA). Peptides were eluted using a gradient from 0.1% trifluoroacetic acid (TFA) to 80% acetonitrile in 0.1% TFA. The composition of the peptides was confirmed by amino acid analysis and sequencing by the Protein Core facility at UTHC.

In this series of studies, only Ac-RRWWCR-NH_2 (SEQ ID NO:1) and QIPRRSWCRFLF (SEQ ID NO:52) inhibited binding of IL-8 to neutrophils (Table 3).

TABLE 3

Inhibition of IL-8 Binding to Neutrophils by Synthetic Peptides

Peptide Tested	SEQ ID NO:	% Binding Inhibition
Ac-KELRCQ	54	-0.4 ± 11.8
QIPRRSWCRFLF	52	61.5 ± 1.0
GWRRWWCDAVLY	53	-12.9 ± 2.9
ELRCQCIKTY	49	7.6 ± 2.1
ELRSQSIKTY	50	5.8 ± 3.9
ELRMQMIKTY	51	-6.9 ± 3.0
Ac-RRWWCR	1	98.6 ± 0.9
RXXXXX	43	11.5
XXXXXR	44	5.8
XRXXXX	45	9.9
XXWXXX	46	1.4
XXXWXX	47	18.5
XXXXCX	48	7.1

EXAMPLE V

INHIBITION OF GRO AND MIP2B NEUTROPHIL BINDING

Ac-RRWWCX-NH_2 (SEQ ID NO:23) was examined for the ability to inhibit other CXC intercrines. The present example demonstrates that, in addition to IL-8 inhibition, Ac-RRWWCX-NH_2 (SEQ ID NO:23) effectively inhibits GRO and MIP2β binding to human neutrophils.

MIP2β and GRO/MGSA were radioiodinated using Bolten Hunter reagent. The radioiodinated components were mixed with various concentration of the Ac-RRWWCX-NH_2 (SEQ ID NO:23) peptide and incubated at room temperature for 15 min. Neutrophil suspension (1×10^6 cells in 160 µl PBS containing 0.1% BSA) was added to 40 µl of the mixture and incubated for 90 min on ice. The radioactivity bound to the cells was separated from free radioactivity by centrifugation through an oil layer. The bound radioactivity is an indication of

bound CXC intercrine peptide. The % binding inhibition in the presence of Ac-RRWWCX-NH_2 (SEQ ID NO:23) was calculated as follows:

$$\% \text{ binding inhibition} = 1 - \frac{\text{B-NSP}}{\text{T-NSP}} \times 100$$

where B is bound radioactivity in the presence of the peptide, T is bound radioactivity in the absence of the peptide, and NSP is bound radioactivity in the presence of excess nonlabeled ligand.

Ac-RRWWCX-NH_2 (SEQ ID NO:23) inhibited binding of 1 nM IL-8 to neutrophils in a dose dependent manner, with an EC_{50} of almost 25 μM. Ac-RRWWCX-NH_2 (SEQ ID NO:23) was also found to effectively suppress the binding of 1 nM GRO and 1 nM MIP2β to neutrophils in a similar manner.

EXAMPLE VI

The following example illustrates the effect of a particular hexapeptide, RRWWCR, on binding of MGSA/GROα to melanoma cells.

In this study, the binding of α-chemokines to Hs 294T melanoma cell line was examined showing that MGSA/GROα but not IL-8 could bind to the cells. This finding is compatible with a previous study in which the presence of a receptor unique for MGSA/GROα on Hs 294T cells was indicated (Horuk *et al.*, 1993). However, studies have shown that IL-8 receptor B is present in melanoma cells and plays a role in the cell growth (Mueller *et al.*, 1994; Norgauer *et al.*, 1996).

IL-8 receptor B messenger RNA and protein expression have been identified in various melanoma cell lines including Hs 294T. The antibody against IL-8 receptor B partially blocked specific binding of MGSA/GROα. Furthermore, addition of $F(ab')_2$ fragments of the anti-IL-8 receptor B or anti-MGSA/GROα monoclonal antibody inhibited serum-independent melanoma cell growth *in vitro* (Norgauer *et al.*, 1996).

These results suggested that, at least a part of, binding of MGSA/GROα to the melanoma cells is mediated by IL-8 receptor B on the surface. As shown in Table 4, melanoma cell lines produced IL-8 at approximately 10 times higher concentration than MGSA/GROα. Therefore, the inability to see IL-8 binding to melanoma cells may result from two types of

α-chemokine receptors on melanoma cells, IL-8 receptor B and a unique receptor for MGSA/GROα, while IL-8 receptor B is completely down-regulated by high concentration of IL-8. However, the presence of a putative receptor has yet to be confirmed. It is also possible that, although IL-8 receptor B rather than a unique MGSA/GROα receptor is expressed on melanoma cells, IL-8 in the culture renders IL-8 receptor B insensitive to IL-8 but not to MGSA/GROα via conformational changes in the receptor. The latter explanation is supported by a report that the interaction of IL-8 receptor B with its legands is not homogeneous (Hayashi *et al.*, 1995).

Methods

Peptide Synthesis

A hexapeptide, RRWWCR with acetylated N-terminus and amidated C-terminus (Antileukinate), was synthesized and purified by Houghten Pharmaceutical Inc. in San Diego as described in Example 1. In some studies, the Antileukinate was radioactively labeled by acetylating the amino terminus with tritiated anhydrous acetic acid. This procedure was carried out at Multiple Peptide Systems (San Diego, CA). The resulting specific radioactivity was 156 Ci/mole.

Cell Culture

Melanoma cell lines, Hs 294T and RPMI-7951, were purchased from American Type Culture Collection (Rockville, MD). A liver cancer cell line, Hep G2, was obtained from M.-C Liu (University of Texas Health Center at Tyler). The cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum (HyClone Laboratories Inc., Loan, UT), 2 mM L-glutamine, 50 unit/ml of penicillin and 50 mg/ml of streptomycin.

Binding Assays

Recombinant human MGSA/GROα and IL-8 were purchased form Pepro Tech Inc. (Rocky Hill, NJ) and radioactively labeled using ^{125}I-labeled Bolton-Hunter reagent (DuPont-NEN, Wilmington, DE) (Bolton and Hunter, 1973). The labeled proteins were isolated using Sephadex G-25 column (Column PD-10, Pharmacia) pre-equilibrated with PBS containing 0.1% gelatin and stored at -70°C after the addition of 1% bovine serum albumin.

Binding studies with melanoma cell lines were performed by the method of Horuk and colleagues (Horuk *et al.*, 1993). Briefly, the cells (1×10^5 cells/well) in 24-well plates were

washed two times with Hank's Balanced Salt Solution and incubated with ^{125}I-labeled MGSA/GROα or IL-8 in the buffer containing 1% bovine serum albumin at 4°C for 3 hr. The incubation was terminated by vacuum aspiration of the supernatant. The cells were washed three times in binding buffer, solubilized by the addition of 200 µl of 1% SDS, and transferred to vials for counting. The non-specific binding was estimated by measuring the binding in the presence of 100-fold excess of non-labeled ligand. The binding parameters were calculated using the Lundon II computer program (Lundon Software Inc., Chagrin Falls, OH).

Measurement of MGSA/GROα and IL-8

HGSA/GROα in the culture supernatants was measured using an enzyme immunoassay (R and D Systems, Minneapolis, MN). IL-8 was measured with a sandwich enzyme immunoassay (Ko *et al.*, 1992). Plates were coated with monoclonal anti-human IL-8 antibody grown and purified from hybridoma HB9647 which was obtained from Dr. E. J. Leonard (Immunopathology Section, Laboratory of Immunobiology, National Cancer Institute, Frederick, MD). Samples were then added and IL-8 bound to the plates was detected with a two step incubation with rabbit polyclonal anti-human IL-8 antibody (Upstate Biotechnology, Lake Placid, NY) and swine anti-rabbit immunoglobulins conjugated with horseradish peroxidase (DAKO Corp., Carpinteria, CA). Both immunoassays were specific for their antigen and did not cross-react with other members of the α-chemokine family.

Analysis of Antileukinate Breakdown

Hs 294T and Hep G2 cells, 2×10^4 cell/well, were cultured in 24 well tissue culture plates for 24 hr. Then the culture media were replaced with media containing tritiated Antileukinate, 0.2 µCi, mixed with 100 µM unlabeled peptide in each well. The cells were then cultured for the indicated periods. The supernatants were collected and stored at -70°C until use. The breakdown of Antileukinate during the cell culture was analyzed on high performance liquid chromatography (HPLC) using an analytical C18 reversed phase column (Waters Co., New Bedford, MA). The peptides were eluted using a gradient from 0.1% trifluoroacetic acid (TFA) to 80% acetonitrile in 0.1% TFA. One milliliter fractions were collected and measured for their radioactivity content using a liquid scintillation counter.

Cell Growth Assays

The cell lines (2×10^4 cells/well) in 500 µl cell culture medium were grown in 24 well tissue culture plates. After the cells adhered to the plate, the supernatants were aspirated and 500 µl of media containing various concentrations of Antileukinate were added to the wells. The cells were cultured for period indicated in the text. The culture media were changed to fresh media every 24 hr. After incubation, the cells were detached with trypsin-EDTA solution (Life Technologies Inc., Gaithersburg, MD, containing 0.05% of trypsin and 0.53 mM of EDTA in Hanks Balanced Salt Solution without Ca^{2+} and Mg^{2+}) and the cell number was counted using a hemocytometer.

Measurement of Cytotoxic Activity of the Peptide

The cell lines (4×10^5 cells/well) in RPMI-1640 medium containing 10% fetal bovine serum were incubated with 10 µCi of $Na_2{}^{51}CrO_4$ (DuPont NEN Co.) for 60 min at 37°C in a 24 well tissue culture plate. The cells were washed 3 times and then incubated in cell culture medium for 30 min at 37°C to allow spontaneous lysis of marginally viable cells. After washing twice, a 200 µl aliquot of the Antileukinate in cell culture medium was added to each well. The cells were cultured for 16 hr at 37°C and then the radioactivity in the supernatants was counted in a gamma radiation spectrometer. Quadriplicate wells received cell culture medium alone or 2% SDS to determine spontaneous and maximum release, respectively. The percentage lysis was calculated by using the following formula:

$$\%Lysis = \frac{(experimental\ cpm\ -\ spontaneous\ cpm)}{(maximum\ cpm - spontaneous\ cpm)} \times 100$$

Statistics

The data are expressed as the mean and standard deviation (S.D.) unless otherwise noted. Significance of differences between multiple groups was tested using the analysis of variance and Scheffe's Test.

Results

The effect of Antileukinate on autostimulatory growth of melanoma cell lines Hs 294T and RPMI-7951 was tested. Antileukinate significantly inhibited the binding of MGSA/GROα to these melanoma cell lines. Since Hs 294T and RPMI-7951 secreted MGSA/GROα into the

supernatant, the effect of Antileukinate on the autostimulatory growth of the cells was examined. When the peptide was added to the culture of melanoma cells, it suppressed the cell growth almost completely at 100 μM. The peptide was more effective on Hs 294T cells than RPMI-7951 cells. The peptide did not affect the growth of the Hep G2 liver cancer cell line, which did not produce MGSA/GROα.

The melanoma cell growth inhibition by Antileukinate seemed to be associated with specific ability of the peptide to inhibit α-chemokine-receptor interaction rather than non-specific cell growth inhibition by itself or its metabolites. Antileukinate did not affect the growth of SP2/o mouse myeloma cell lines stimulated by murine interleukin-6. Antileukinate did not inhibit the growth of Hep G2 cells which metabolized the peptide in a similar way to Hs 294T cells. The cell viability of Hs 294T in the presence of 100 μM peptide was greater than 92% as assessed by trypan blue dye exclusion. The peptide did not cause cell lysis of either melanoma cell lines or Hep G2 cells. Furthermore, the removal of the peptide from Hs 294T cell culture or the addition of excess MGSA/GROα to the culture restored the cell growth. This suggested that MGSA/GROα is an essential autostimulatory growth factor for melanoma cells and Antileukinate inhibits their growth by preventing MGSA/GROα from binding to its receptors.

TABLE 4

IL-8 and MGSA/GROα in the Culture Supernatants

	origin of cell line	IL-8* (ng/ml)	MGSA/GROα* (ng/ml)
Hs 294T	melanoma	178.3 ± 1.0	24.3 ± 0.8
RPMI-7951	melanoma	138.2 ± 0.7	2.6 ± 0.1
Hep G2	liver	2.0 ± 0.0	<0.06

* The cells (5×10^4) were cultured in a 24 well plate for 48 hr. The supernatants were collected and the concentrations of chemokines were measured as described in the text.

TABLE 5

Cytotoxic Test of Antileukinate on Cultured Cell Lines

Peptide Added	% cell lysis*		
(μM)	Hs 294T	RPMI-7951	Hep G2
0	0.0 ± 4.5	0.0 ± 2.7	0.0 ± 11.0
1	-4.0 ± 5.2	-0.8 ± 2.2	-2.1 ± 7.5
5	-3.2 ± 7.2	-2.6 ± 3.7	-0.8 ± 8.3
20	-1.6 ± 3.7	-3.4 ± 6.3	5.8 ± 8.3
100	2.4 ± 7.9	6.8 ± 11.1	0.9 ± 2.9

* The cells labeled with ^{51}Cr were cultured in the presence of various concentrations of Antileukinate for 16 hr. Then the radioactivity in the supernatants was counted. The percentage cell lysis was calculated based on the amount of chromium released from the cells during the culture.

Effect of Antileukinate on Binding of MGSA/GROα to Melanoma Cells

The binding of radiolabeled MGSA/GROα to melanoma cell lines was specific and saturable (FIG. 11). The Kd values calculated were approximately 6×10^{-9} M and 3×10^{-9} M for Hs 294T and RPMI-7951, respectively. The maximal binding (B_{max}) of MGSA/GROα to the melanoma cell lines was 11 fmole/10^5 cells and 15 fmole/10^5 cells respectively. Thus, the number of MGSA/GROα binding sites were estimated to be 70,000 - 100,000/a melanoma cell. There was, however, no specific binding of IL-8 to either cell line (FIG. 9).

Antileukinate inhibited the binding of MGSA/GROα to both of the melanoma cell lines. When the melanoma cell lines, Hs 294T and RPMI-7951, were incubated with ^{125}I-labeled MGSA/GROα, 1 nM, in the presence of Antileukinate, the binding was inhibited by Antileukinate with an EC_{50} of approximately (1 μM (FIG. 10).

The Production of α-chemokines form the Melanoma Cell Lines

The supernatants of melanoma cells cultured for 48 hr were tested for the presence of α-chemokines. The concentration of MGSA/GROα was 24.3 ± 0.8 ng/ml in Hs 294T and 2.6 ±

0.1 ng/ml in RPMI-7951 supernatants (Table 4). IL-8 was detected at much higher concentrations in the supernatants from both melanoma cell lines (Table 4). Liver cancer cell line, Hep G2, also produced detectable amounts of IL-8, however, the concentration was only a small fraction of that seen in the melanoma cell cultures. MGSA/GROα could not be detected in the supernatants of Hep G2 cells.

Effect of Antileukinate on Melanoma Cell Growth

Since Antileukinate inhibited the binding of MGSA/GROα to melanoma cell lines, the ability of Antileukinate to suppress auto-stimulatory growth of melanoma cells was tested. Prior to these studies, the time dependent breakdown of Antileukinate by Hs 294T melanoma cells and Hep-G2 liver cancer cells were examined. When the radiolabeled Antileukinate in RPMI-1640 medium was analyzed by HPLC, the major peak of the radioactivity was eluted at 50-51 ml (FIG. 14). After a 24 hr culture period, the major peak had decreased by more than 85% and there was a transient increase in the peek seen at 41-42 ml (FIG. 14). These findings showed that most Antileukinate in both cell cultures disappeared within 24 hr by a two step catabolic process. Therefore, in later studies the culture medium containing Antileukinate was exchanged every 24 hr to maintain the effect of Antileukinate.

Cell lines were cultured in the presence of various concentrations of Antileukinate for 72 hr and the number of cells in the well were counted. The growth of Hs 294T cells was significantly inhibited by 10 μM peptide (FIG. 12A) with an ED_{50} of 18 μM. Antileukinate also suppressed the growth of RPMI-7951 melanoma cells with an ED_{50} of 31 μM (FIG. 12B); however, it did not affect the growth of Hep G2 liver cancer cells (FIG. 12C).

The inhibition of Hs 294T growth was further characterized. It was reversed by the removal of Antileukinate from the culture media (FIG. 13A) and by the addition of 50 nM MGSA/GROα to the receptors on the Hs 294T cell surface.

Cytotoxicity of Antileukinate

To exclude the possible contribution of cytotoxicity of Antileukinate to the inhibition of melanoma cell growth, ^{51}Cr-labeled cells were cultured in the presence of the peptide for 16 hr. Antileukinate did not cause cell lysis of either of the melanoma cell lines or Hep G2 cell line (Table 5). Measurements of cell viability by Trypan Blue exclusion also indicated that

Antileukinate was not toxic to the cells (FIG. 14). The adhesivity of Hs 294T, RPMI-7951 and Hep G2 cells to the culture wells did not alter when Antileukinate was added.

Effect of Antileukinate on other Tumor Cells

Antileukinate was shown to inhibit the growth of a wide range of tumor cells in addition to melanoma cells. FIG. 15 illustrates a decrease in cell number of adenocarcinoma of prostate cells. The effect is dose dependent. Similar effects are observed at even lower concentrations of Antileukinate on squamous cell carcinoma cells (NCI-H292) from lung (see FIG. 16). FIG. 17 shows the effect of antileukinate on growth of A549 cells (from adenocarcinoma of the lung).

EXAMPLE VII

Materials and Methods

Cell Culture. Human melanoma cell lines, RPMI7951, Hs294T, A375, WM115, C32, SK-MEL-2, SK-MEL-3, SK-MEL-24 and SK-MEL-28 were purchased from American Type Culture Collection (Rockville, MD). A375SM, C8161-P, amelanotic and highly metastatic human melanoma cell lines, and C8161-C, melanotic melanoma cell line, were kindly provided by Dr. I. J. Fidler (University of Texas M.D. Anderson Cancer Center), Dr. D. R. Welch (Pennsylvania State University College of Medicine), and Dr. F. L. Meyskens (University of California, Irvine) (Bregman, et al., 1983; Gutman, et al., 1995; Lee, et al., 1996), respectively. Normal melanocyte cells were purchased from Clonetics (San Diego, CA). The cell lines were maintained in culture as adherent monolayers in RPMI 1640 (RPMI7951 and Hs294T) or MEM (A375, A375SM, WM115, C32, SK-MEL-2, SK-MEL-3, SK-MEL-24 and SK-MEL-28) supplemented with 10% FCS (Sigma Chemical Co., St. Louis, MO). C8161-C and C8161-P were maintained in DME / Ham's F-12 (GIBCO) supplemented with 5% and 10% FCS, respectively. Normal human melanocytes were maintained in Melanocyte Growth Medium supplemented with 2% FCS, 1 ng/ml human fibroblast growth factor-B, 15 μg/ml bovine pituitary extract, and 5 μg/ml bovine insulin (Clonetics). The cells were passed when they reached 70-80% confluence and maintained for no longer than 4 weeks. All cell culture was performed at 37 ^{o}C in a 5% CO_2 atmosphere.

Peptide Synthesis. A hexapeptide, RRWWCR with acetylated N-terminus and amidated C-terminus, Antileukinate, was synthesized and purified by Multiple Peptide Systems (San Diego, CA) as described previously (Hayashi, et al., 1997; Hayashi, et al., 1995).

Quantitation of GROα and IL-8. GROα accumulation in the culture supernatants was quantitated using a commercially available ELISA (R & D Systems, Minneapolis, MN). IL-8 was quantitated using an ELISA as previously described (Sylvester, et al., 1990; Miller, et al., 1996). The assay employed a monoclonal murine IgG_1 Ab purified from ascites developed with hybridoma HB9647 (Sylvester, et al., 1990) (ATCC, with permission from Dr. E. J. Leonard), and a rabbit polyclonal anti-human IL-8 polyclonal antiserum (Upstate Biotechnology Inc., Lake Plasid, NY) and swine anti-rabbit immunogloblins conjugated with horseradish peroxidase (DACO, Carpinteria, CA). Both immunoassays were specific for their antigen and did not cross-react with other members of the α-chemokine family (Miller, et al., 1996).

Assessment of Cell Proliferation. A375SM and C8161-C cell lines were plated at a density of 5×10^4 cells/well in a 96-well plate and cultured in growth medium for 4 hours. The medium was then aspirated and replaced with growth medium without FCS and the cells were cultured for a further 16 hours. The indicated concentrations of anti-GROα mAb, anti-IL-8 mAb (R & D Systems, Minneapolis, MN), human *r*GROα (Pepro Tech, Rocky Hill, NJ) or Antileukinate were replaced in the wells and the number of living cells was evaluated by the MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma Chemical Co.) assay after 24 hours (Norgauer, et al., 1996; Mosmann, 1983). In these experiments, the absorption at 570 nm was measured and the number of cells were expressed as a percentage of cells cultured in control media.

Binding Assays. Human *r*GROα was radioactively labeled using ^{125}I-labeled Bolton-Hunter reagent (DuPont-NEN, Wilmington, DE) (31). The labeled protein was isolated using PD-10 columns (Pharmacia, Piscataway, NJ) pre-equilibrated with PBS containing 0.1% gelatin. The ^{125}I-GROα stored at -70°C after the addition of 1% BSA.

Binding studies with melanoma cell lines were performed as previously described (13). Briefly, melanoma cells were plated in growth medium at a density of 2×10^4 cells/well

in a 96-well plate and cultured for 16 hours. The cells were then washed two times with HBSS (GIBCO) and incubated with 1 nM radiolabeled GROα or IL-8, and Antileukinate (0-500 μg/ml) diluted in 1% BSA at 4°C for 3 hours. The incubation was terminated by aspiration of the supernatant. The cells were washed three times in binding buffer, solubilized by the addition of 200 μl of 1% SDS (EM SCIENCE, Cherry Hill, NJ), and transferred to vials for counting. The percentage binding inhibition was calculated by using the formula:

$$\% \text{ BINDING INHIBITION} = \frac{\text{TOTAL CPM - EXPERIMEENTAL CPM}}{\text{TOTAL CMP}}$$

The binding parameters were calculated using the Lundon II computer program (Lundon Software, Chagrin' Falls, OH).

Measurement of Cytotoxic Activity of the Peptide. The cells were plated at a density of 4 × 10^5 cells/well in a 96-well plate and incubated with 2 μCi/well of $Na2^{51}CrO_4$ (DuPont-NEN, Wilmington, DE) overnight at 37°C. Following the incubation, the cells were washed 3 times and incubated in culture medium for a further 30 minutes at 37°C to allow spontaneous lysis of marginally viable cells. After washing twice, a 200 μl aliquot of Antileukinate in culture medium was added to each well. The cells were cultured for 24 hours at 37°C and then the radioactivity in the supernatants was counted using a gamma radiation spectrometer. Quadruplicate wells received culture medium alone or 2% SDS to determine spontaneous and maximum release, respectively. The percentage lysis was calculated by using the formula:

$$\% \text{ LYSIS} = \frac{\text{EXPERIMENTAL CPM-SPONTANEOUS CPM}}{\text{MAXIMUM CPM - SPONTANEOUS CPM}} \times 100$$

Tumor Growth Assay. All procedures had been approved, in advance, by the institution animal care and use committee at the University of Texas Health Center at Tyler. Female, 3- to 4-week-old, athymic BALB/c nude mice were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). The mice were housed in laminar flow rooms under specific pathogen-free

conditions. A375SM cells were harvested using 0.53 mM EDTA-0.05% trypsin (GIBCO). The flask was tapped sharply to dislodge the cells, supplemented medium was added, and the cell suspension was pipetted again to produce a single cell suspension. The cells were then washed and resuspended in ice-cold Ca^{2+}- and Mg^{2+}-free HBSS to a concentration of 1×10^6 cells / 100 µl and cell viability was determined by trypan blue exclusion. Only cultures with >95% viability were used. The cells were then implanted subcutaneously in the midline dorsum of each unanesthetized mouse in a volume of 100 µl. Tumor size was measured by taking orthogonal measurements and were expressed as mean tumor diameter. Mean tumor diameter was calculated as described before (Welch, et al., 1991) by using the formula:

$$\text{MEAN TUMOR DIAMETER} = (\text{DIAMETER}_X) \text{ X } (\text{DIAMETER}_Y)$$

After 1 week post-implantation, Micro-Osmotic Pump (Alza Co., Palo Alto, CA), which administered 125 µg/hour/body of Antileukinate in saline or saline alone as a control, for 7 days, were implanted in mice following anesthesia by methoxyflurane (Mallinckrodt Veterinary, Inc., Mundelein, IL).

Pulmonary Metastasis Assay. Female, 3- to 4-week-old, athymic BALB/c nude mice were purchased and housed same as above. A375SM cells were harvested to the concentration of 5×10^5 cells / 100 µl and cell viability was determined same as above. The cells (100 µl) were then injected into the lateral tail vain of unanesthetized mice. After 1 week post-inoculation, Micro-Osmotic pump which administered 125 µg/hour/body of Antileukinate in saline or saline alone as a control for 14 days, were implanted to mice following methoxyflurane anesthesia. After 4 weeks post-inoculation, mice were exsanguinated by cardiac puncture following full anesthesia. Lungs were removed and fixed in 3.7% formaline with 4% NaH_2PO_4 and 1.2% Na_2HPO_4 in H_2O. The colonies of the lung surface were counted with the aid of a dissecting microscope (Welch, et al., 1991; Miele, et al., 1994).

A375SM, C8161-P (highest GROα and lowest IL-8 accumulation in the culture supernatants), Hs294T (one of the highest accumulation of both GROα and IL-8) and WM115 (the accumulation of both GROα and IL-8 were closed to A375SM) cells were harvested and injected same as above without implanting pump. After 4 weeks post-inoculation, mice were

exsanguinated, lungs were removed, fixed, and the colonies on the surface of lung were enumbered as above.

Statistics. The data are expressed as the mean and SD unless otherwise noted. Significance of differences between two groups were tested using the analysis of Student's t-test. Data were considered statistically significant if p values were £ 0.05. The experiments were usually performed at least twice with at least four replicate cultures per experiment.

Results

The Secretion of GROα and IL-8 from Melanoma Cell Lines and Melanocytes. To study the production of GROα and IL-8 by melanoma cell lines and normal human melanocytes, the accumulation of these chemokines was measured in the culture supernatants using an ELISA. Supernatants from cells cultured for 48 hours were tested for the presence of GROα and IL-8 (Table 1). GROα and IL-8 were detected in supernatants from all of the cell lines. The concentrations of GROα ranged between 42.0 ± 21.3 and 4988.2 ± 244.5 pg/ml and IL-8 ranged between 37.5 ± 65.0 and 16351.8 ± 5375.4 pg/ml. Interestingly, normal melanocytes also produced both GROα and IL-8, and their concentrations were higher than some melanoma cell lines.

The Effect of Neutralizing Antibodies and Human rGROα on Melanoma Cell Growth. We tested the ability of anti-GROα mAb or anti-IL-8 mAb to suppress and human *r*GROα to stimulate the growth of A375SM and C8161-C cell lines. The cell number was decreased in a dose dependent manner and there are significant difference between anti-GROα mAb and control Ab at concentrations greater than, or equal to 20 μg/ml (A375SM) and 50 μg/ml (C8161-C), whereas anti-IL-8 mAb was ineffective (Fig. 1). Furthermore, the addition of human *r*GROα accelerated the proliferation of A375SM and C8161-C cell lines in a dose-dependent manner. There were significant differences from control culture (culture medium alone) at concentrations greater than, or equal to 50 nM of GROα.

The Effect of Antileukinate on the Binding of GROα to Melanoma Cell Lines and Melanocytes. Antileukinate inhibited the binding of GROα to melanoma cell lines and

melanocytes. When A375SM and C8161-C cells were incubated with 1 nM of ^{125}I-labeled GROα in the presence of Antileukinate, the binding was inhibited ID_{50} of 13.6 and 6.1 μg/ml, respectively. Binding studies with the other melanoma cell lines and melanocytes were performed and similar inhibition curves to those of A375SM and C8161-C cells were obtained (data not shown). The ID_{50} ranged between 1.0 to 13.6 μg/ml..

The Effect of Antileukinate on the Growth of Melanoma Cell Lines. Antileukinate was tested for its ability to suppress auto-stimulatory growth of A375SM and C8161-C cell lines. The cells were cultured in the presence of various concentrations of Antileukinate for 24 hours, after which time the number of cells in each well was counted. The cell numbers were decreased in a dose dependent manner in the presence of Antileukinate and there were significant differences from control cultures in the absence of Antileukinate at concentrations greater than, or equal to 50 μM (A375SM) and 20 μM (C8161-C) of Antileukinate.

Cytotoxicity of Antileukinate. To exclude the possible contribution of cytotoxicity to the Antileukinate inhibition of A375SM and C8161-C cell growth, we cultured ^{51}Cr-labeled cells in the presence of the peptide for 24 hours. Antileukinate did not cause significant cell lysis of A375SM or C8161-C cell lines (Table 2).

The Effect of Antileukinate on Tumor Growth. A375SM-implanted mice were maintained with continuous administration of saline alone (control) or Antileukinate in saline for 7 days after inoculation. Mean tumor diameter was calculated 3 times per week. After continuous administration of Antileukinate (n=17), tumor growth was significantly inhibited by the 11th day and compared to saline group (n=18). Treatment with Antileukinate led to an inhibition 59.0% of mean tumor diameter at 14 days after inoculation. There was no critical adverse effects noted during the 14 days.

Production of Pulmonary Metastasis. The mice, into which Hs294T, WM115, A375SM, or C8161-P (this cell line was thought to be different from C8161-C cell line from its color and producing pattern of GROα and IL-8) cells had been injected, were maintained for 28 days and after which time, the lungs were removed, fixed and colonies on the surface of the

lung were counted. The A375SM-injected group (n=10) and C8161-P-injected group (n=4) developed pulmonary metastasis and the number of colonies were between 8 to 529 and 149 to 453 per mouse, respectively. On the other hand, no pulmonary metastasis were detected in either the Hs294T- (n=14) or WM115-injected (n=12) group. Statistical analysis showed that there were significant differences between the A375SM, C8161-P groups, and Hs294T, WM115 groups ($p<0.0001$).

The Effect of Antileukinate on Pulmonary Metastasis. Mice were injected with A375SM and after 7 days were treated with continuous administration of Antileukinate in saline or saline alone. This treatment was maintained for further 14 days. Twenty eight days after inoculation, the lungs were removed, fixed and the colonies on the surface of the lung were counted. There was no critical adverse effects noted during the period of Antileukinate administration. The number of lung colonies were significantly reduced in the Antileukinate treated group (n=9) compared to the control group (n=11) ($p=0.0134$). Treatment with Antileukinate led to a mean reduction of 60.3 % in the number of lung colonies which had developed.

DISCUSSION

Melanoma is one of the most common malignancies, and more than 40,000 new cases and 7,300 deaths were reported in the in the United States in 1996 . Despite attempts to advance early diagnosis and the use of many combination chemotherapy regimens for patients with advanced tumors, the prognosis is still poor . Although the adjuvant therapy with high-dose interferon α-2β has significant impact on remission and overall survival, the side effects associated with this regimen are moderate to severe, leading to a need to seek other modes of treatment.

The autocrine secretion of growth factors appears to be a central mechanism in the process of malignant transformation, and it has been reported that proliferation of melanoma is regulated by GROα (Hayashi, *et al.* 1997). GROα and IL-8, members of the superfamily of α-chemokines, have specific receptors on the target cell surface. In particular CXCR1 and CXCR2, receptors for α-chemokines, are expressed in a variety cells. The function of these chemokines depends on the interaction between the ligand and its specific receptor.

It has been demonstrated that all of the melanoma cells tested produced and secreted GROα and IL-8. Binding of exogenous GROα by A375SM and C8161-C cells stimulated the

cell proliferation supporting the notion that GROα is a growth stimulating factor for these cells. Addition of anti-GROα mAb to the culture inhibited the proliferation of A375SM and C8161-C cells indicating that GROα is also an essential growth factor for these cells. IL-8 has been shown to be angiogenic and it stimulates the lung cancer cell tumor growth (Arenberg, *et al.*, 1996). Inhibition of IL-8 reduces lung cancer tumor growth (Koch, *et al.*, 1992). Additionally, IL-8 has been reported to be a growth factor for some melanoma cell lines (Schadendorf, *et al.*, 1993; Singh, *et al.*, 1994). Although the proliferation of A375SM and C8161-C cells were not inhibited by anti-IL-8 mAb, IL-8 may be involved in the growth of melanoma tumors as an angiogenic factor.

Previous studies have shown that Antileukinate competitively inhibited the binding of GROα and IL-8 to the receptor on neutrophils and melanoma cell lines in a dose-dependent manner (Hayashi, *et al.*, 1997; Hayashi, *et al.*, 1995). Additionally, the peptide bound to the receptor with high affinity and the activity of the peptide was specific for α-chemokines . In the present study, Antileukinate inhibited the binding of radiolabeled GROα to all melanoma cell lines tested. When A375SM and C8161-C cells were cultured in the presence of Antileukinate, the proliferation of the cells was inhibited in a dose-dependent manner *in vitro*.

The production of metastasis involves a complex sequence of events including cell migration, cell adhesion, cell proliferation at a secondary site, and angiogenesis. In an analysis of 13 human melanoma cell lines, Singh and colleagues showed that expression of IL-8 correlates with the metastatic potential of melanoma cells (Singh, *et al.*, 1994. In the present study, Hs294T cells produced and secreted more of both IL-8 and GROα than the other melanoma cell lines, and the accumulation of IL-8 and GROα in the culture supernatants of WM115 cells was close to that of A375SM, a highly metastatic cell line. Furthermore, C8161-P, a highly metastatic cell line, accumulated high concentrations of GROα but low concentrations of IL-8 in the culture medium. A pulmonary metastasis model involved injecting Hs294T and WM115 cell lines into athymic BALB/c nude mice. No colonies on the pulmonary surface were detected; however, many metastatic colonies were detected when A375SM and C8161-P cells were inoculated. The data indicate that the existence of factors other than GROα or IL-8 may be associated with metastatic potential.

On the other hand, many pulmonary metastatic colonies were detected when A375SM cells were inoculated, and continuous administration of Antileukinate inhibited the metastasis of the melanoma cells in athymic BALB/c nude mice. Continuous administration of Antileukinate

also inhibited the tumor growth of A375SM cells. These data confirm *in vitro* studies with respect to correlation between the metastatic potential and the accumulation of α-chemokines such as IL-8 and GROα. The data indicate that Antileukinate, α-chemokine receptor inhibitor, has strong potential as therapeutic agents for advanced malignant melanoma tumor growth and metastasis.

It has been demonstrated that GROα is an essential autocrine growth stimulating factor and Antileukinate inhibits the growth of melanoma cells by inhibiting the binding of GROα to the receptor both *in vitro* and *in vivo*. While existence of other factors besides GROα and IL-8 that enhance the metastasis, the results herein disclosed, lead to a reasonable expectation that Antileukinate or a derivative of the this molecule may have an important role in the treatment of malignant melanoma.

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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(iii) NUMBER OF SEQUENCES: 58

(iv) COMPUTER READABLE FORM:
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(A) APPLICATION NUMBER: US 08/865,471
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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Arg Arg Trp Trp Cys Arg
1 5

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Arg Arg Trp Trp Cys Arg Xaa
1 5

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Arg Arg Trp Trp Cys Arg Ala
1 5

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Arg Arg Trp Trp Cys Arg Cys
1 5

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Arg Arg Trp Trp Cys Arg Asp
1 5

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Arg Arg Trp Trp Cys Arg Glu
1 5

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Arg Arg Trp Trp Cys Arg Phe
1 5

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Arg Arg Trp Trp Cys Arg Gly
1 5

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Arg Arg Trp Trp Cys Arg His
1 5

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Arg Arg Trp Trp Cys Arg Ile
1 5

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

Arg Arg Trp Trp Cys Arg Lys
1 5

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Arg Arg Trp Trp Cys Arg Leu
1 5

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

Arg Arg Trp Trp Cys Arg Met
1 5

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

Arg Arg Trp Trp Cys Arg Asn
1 5

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

Arg Arg Trp Trp Cys Arg Pro
1 5

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

Arg Arg Trp Trp Cys Arg Gln
1 5

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

Arg Arg Trp Trp Cys Arg Arg
1 5

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

Arg Arg Trp Trp Cys Arg Ser
1 5

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

Arg Arg Trp Trp Cys Arg Thr
1 5

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

Arg Arg Trp Trp Cys Arg Val
1 5

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

Arg Arg Trp Trp Cys Arg Trp
1 5

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

Arg Arg Trp Trp Cys Arg Tyr
1 5

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

Arg Arg Trp Trp Cys Xaa
1 5

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

Arg Arg Trp Trp Cys Ala
1 5

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

Arg Arg Trp Trp Cys Cys
1 5

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

Arg Arg Trp Trp Cys Asp
1 5

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

Arg Arg Trp Trp Cys Glu
1 5

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

Arg Arg Trp Trp Cys Phe
1 5

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

Arg Arg Trp Trp Cys Gly
1 5

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

Arg Arg Trp Trp Cys His
1 5

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

Arg Arg Trp Trp Cys Ile
1 5

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

Arg Arg Trp Trp Cys Lys
1 5

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

Arg Arg Trp Trp Cys Leu
1 5

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

Arg Arg Trp Trp Cys Met
1 5

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

Arg Arg Trp Trp Cys Asn
1 5

(2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

Arg Arg Trp Trp Cys Pro
1 5

(2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

Arg Arg Trp Trp Cys Gln
1 5

(2) INFORMATION FOR SEQ ID NO: 38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

Arg Arg Trp Trp Cys Ser
1 5

(2) INFORMATION FOR SEQ ID NO: 39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

Arg Arg Trp Trp Cys Thr
1 5

(2) INFORMATION FOR SEQ ID NO: 40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

Arg Arg Trp Trp Cys Val
1 5

(2) INFORMATION FOR SEQ ID NO: 41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:

Arg Arg Trp Trp Cys Trp
1 5

(2) INFORMATION FOR SEQ ID NO: 42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:

Arg Arg Trp Trp Cys Tyr
1 5

(2) INFORMATION FOR SEQ ID NO: 43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 43:

Arg Xaa Xaa Xaa Xaa Xaa
1 5

(2) INFORMATION FOR SEQ ID NO: 44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 44:

Xaa Xaa Xaa Xaa Xaa Arg
1 5

(2) INFORMATION FOR SEQ ID NO: 45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 45:

Xaa Arg Xaa Xaa Xaa Xaa
1 5

(2) INFORMATION FOR SEQ ID NO: 46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 46:

Xaa Xaa Trp Xaa Xaa Xaa
1 5

(2) INFORMATION FOR SEQ ID NO: 47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 47:

Xaa Xaa Xaa Trp Xaa Xaa
1 5

(2) INFORMATION FOR SEQ ID NO: 48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 48:

Xaa Xaa Xaa Xaa Cys Xaa
1 5

(2) INFORMATION FOR SEQ ID NO: 49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 49:

Glu Leu Arg Cys Gln Cys Ile Lys Thr Tyr
1 5 10

(2) INFORMATION FOR SEQ ID NO: 50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 50:

Glu Leu Arg Ser Gln Ser Ile Lys Thr Tyr
1 5 10

(2) INFORMATION FOR SEQ ID NO: 51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 51:

Glu Leu Arg Met Gln Met Ile Lys Thr Tyr
1 5 10

(2) INFORMATION FOR SEQ ID NO: 52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 52:

Gln Ile Pro Arg Arg Ser Trp Cys Arg Phe Leu Phe
1 5 10

(2) INFORMATION FOR SEQ ID NO: 53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 53:

Gly Trp Arg Arg Trp Trp Cys Asp Ala Val Leu Tyr
1 5 10

(2) INFORMATION FOR SEQ ID NO: 54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 54:

Lys Glu Leu Arg Cys Gln
1 5

(2) INFORMATION FOR SEQ ID NO: 55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 55:

Ser Tyr Ser Met Glu His Phe Arg Trp Gly Lys Pro Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 56:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 56:

Arg Arg Trp Trp Cys
1 5

(2) INFORMATION FOR SEQ ID NO: 57:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 57:

Arg Arg Trp Trp Cys Xaa Xaa
1 5

(2) INFORMATION FOR SEQ ID NO: 58:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 58:

Arg Arg Xaa Trp Cys Xaa
1 5

CLAIMS

1. A method of inhibiting binding of an α-chemokine to its cognate neutrophil receptor, comprising contacting a neutrophil with a peptide comprising the amino acid sequence Arg Arg Trp Trp Cys Xaa_1 (SEQ ID NO:23) wherein Xaa_1 is any amino acid and the amount of peptide is effective to inhibit binding of the α-chemokine to its cognate neutrophil receptor.

2. The method of claim 1 wherein the α-chemokine is IL-8, MIP-2β, MGSA/Groα, PBP, IP10, PF4, ENA78 or MGSA/Groα.

3. The method of claim 1 wherein the peptide has the amino acid sequence of SEQ ID NO:1

4. The method of claim 1 wherein the peptide has the amino acid sequence of SEQ ID NO:24, SEQ ID NO: 25; SEQ ID NO:26; SEQ ID NO:27; SEQ ID NO:28; SEQ ID NO:29; SEQ ID NO:30 or SEQ ID NO:31.

5. A method of inhibiting binding of an α-chemokine to a tumor cell, comprising administering to said cell an amount of a peptide comprising the sequence of SEQ ID NO:1 effective to inhibit binding of said α-chemokine.

6. The method of claim 5 wherein the tumor cell is identified as an endothelial cell.

7. The method of claim 6 wherein the endothelial cell is a human umbilical vein endothelial cell.

8. The method of claim 5 wherein the tumor cell is a melanoma cell line.

9. The method of claim 8 wherein the melanoma cell line is Hs 294T, RPMI-7951, A375P, A375SM, C8161 or WM115.

10. The method of claim 5 wherein the tumor cell is an adenocarcinoma cell.

11. The method of claim 10 wherein the adenocarcinoma cell is lung adenocarcinoma cell A549, NCI-H441 or SK-LU-1.

12. The method of claim 5 wherein the tumor cell is a squamous lung cancer cell.

13. The method of claim 12 wherein the squamous cell lung cancer cell is NCI-H292.

14. The method of claim 10 wherein the adenocarcinoma cell is selected from the group consisting of stomach AGS, stomach Hs746T, breast cell MCG-7, prostate DU145 and colon Caco-2.

15. A method of inhibiting proliferation of a melanoma cell, comprising inhibiting binding of MGSA/Groα to said cell with an effective amount of a peptide having the amino acid sequence of Arg Arg Trp Trp Cys Arg (SEQ ID NO:1).

16. The method of claim 15 wherein the melanoma cell is in a mammal.

17. The method of claim 15 wherein the growth inhibiting is *in vitro*.

18. The method of claim 17 wherein the *in vitro* growth inhibiting effective amount is defined as between about 40 to about 125 μM or between about 50 μM and about 100μM of the peptide having the amino acid sequence of SEQ ID NO:1.

19. The method of claim 1, wherein the peptide is an acylated peptide or is amidated at the C-terminus or acetylated at the N-terminus and amidated at the C-terminus.

20. The method of claim 1, wherein the peptide comprises D-amino acids or L-amino acids.

21. A method of inhibiting tumor growth comprising administering an antileukinate peptide into or in proximity of the tumor.

22. The method of claim 21 wherein the antileukinate is a peptide having the amino acid sequence of SEQ ID NO:1.

23. Use of a composition comprising an antileukinate compound for use as a medicament.

24. The use of the antileukinate compound of claim 23 which is a peptide having the amino acid sequence of SEQ ID NO:1.

25. Use of the composition of claim 23 for the manufacture of a medicament for the treatment of tumor cell proliferation.

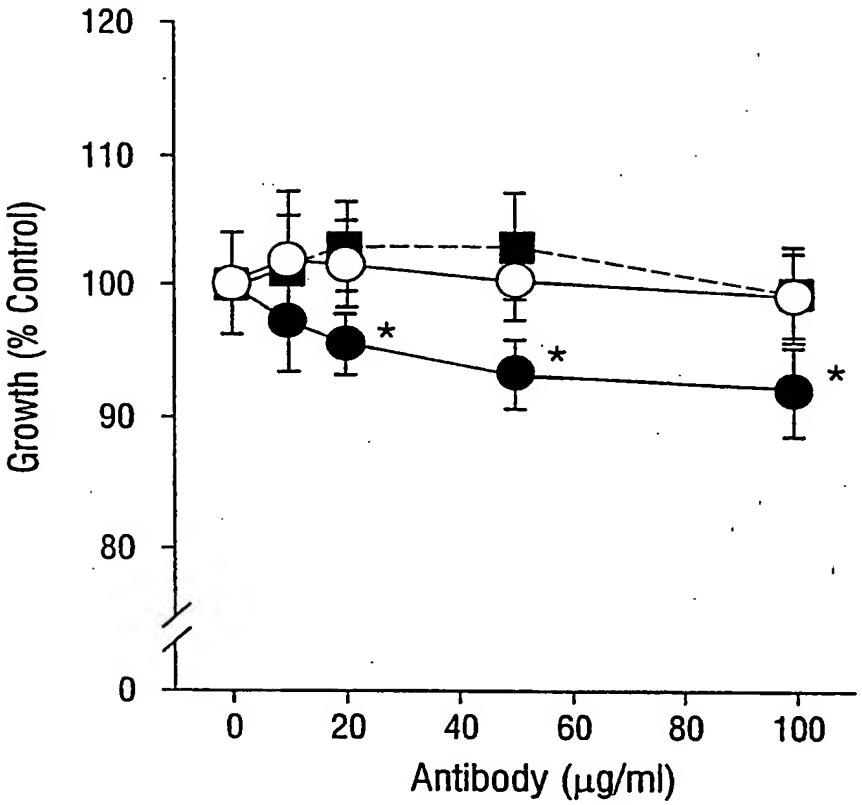

FIG. 1A

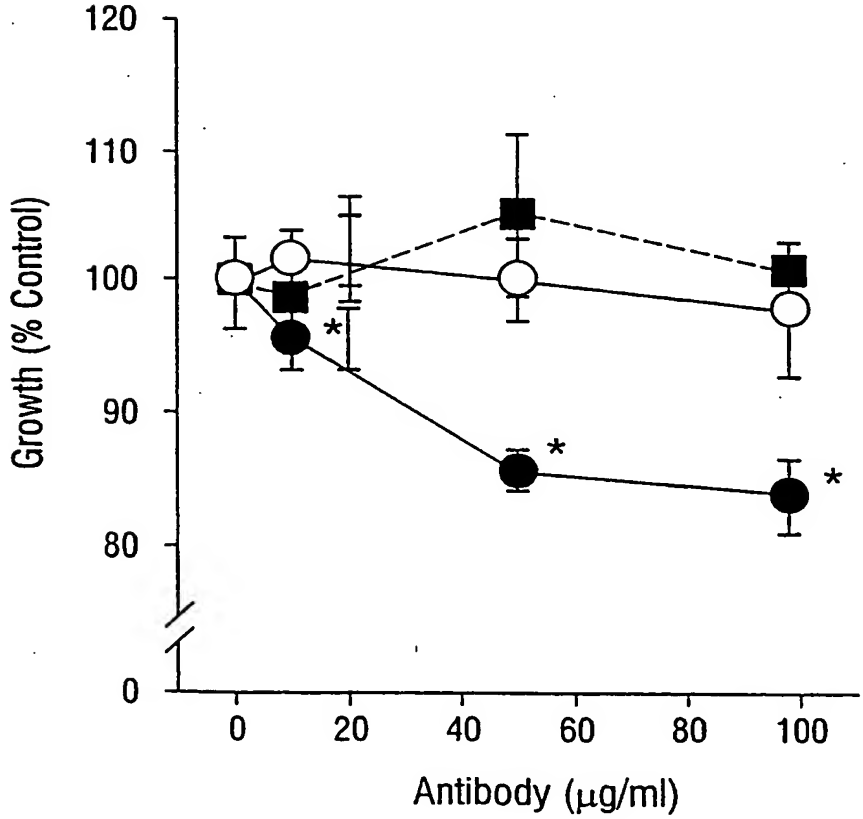

FIG. 1B

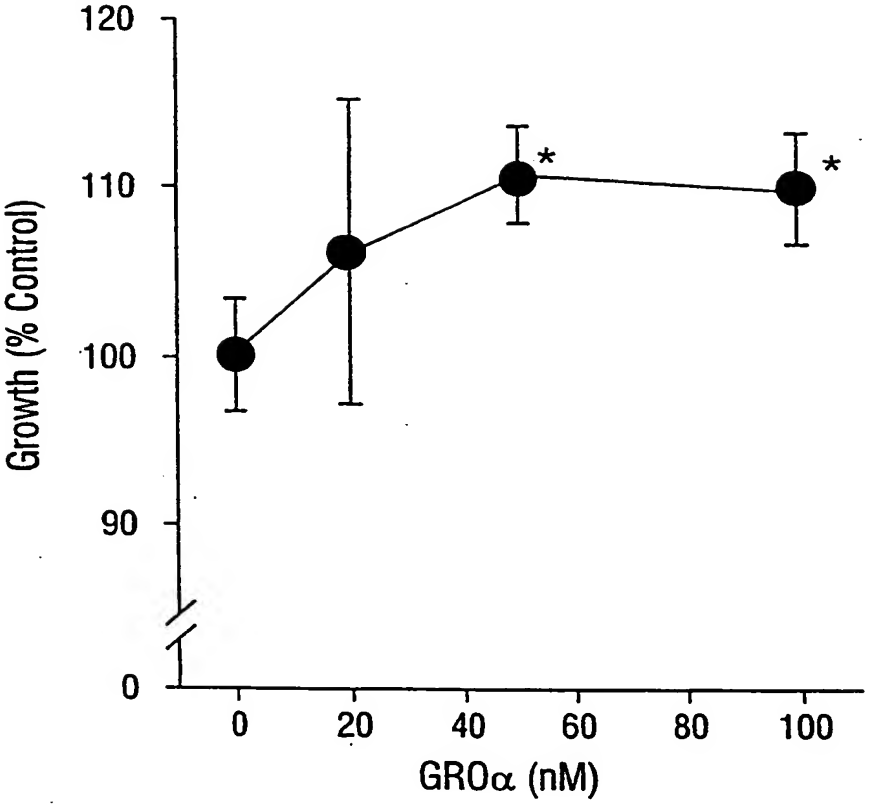

FIG. 2A

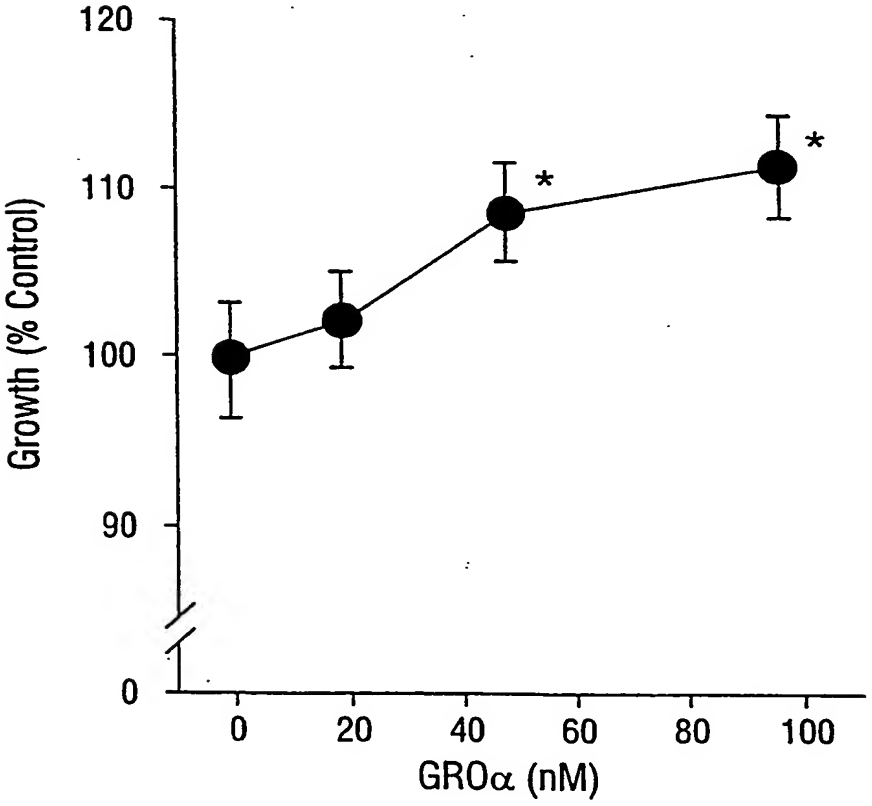

FIG. 2B

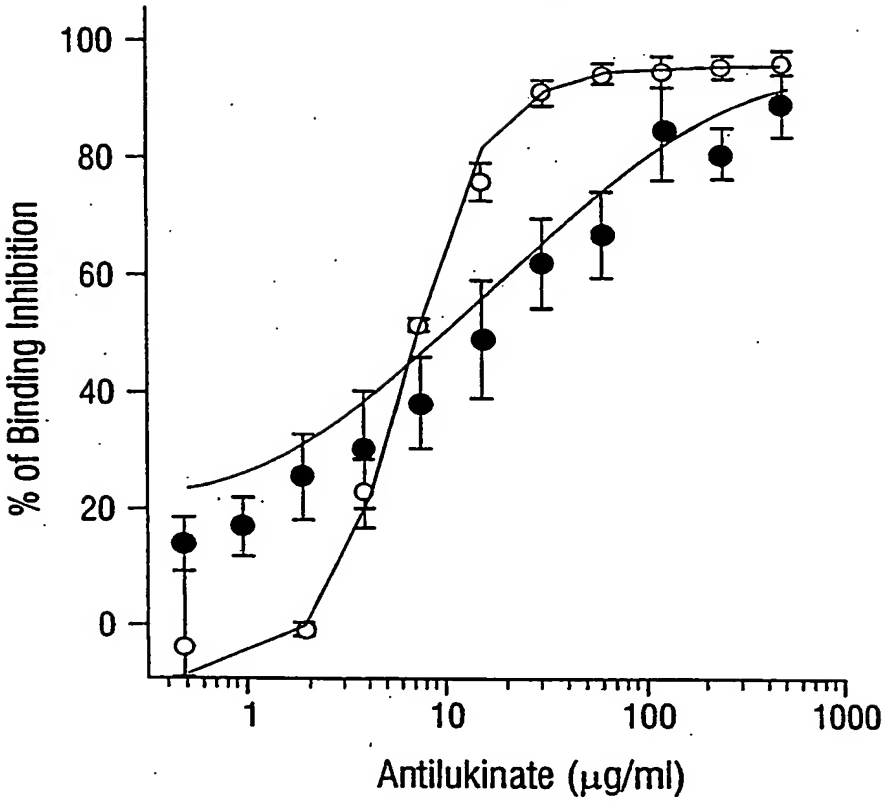

FIG. 3A

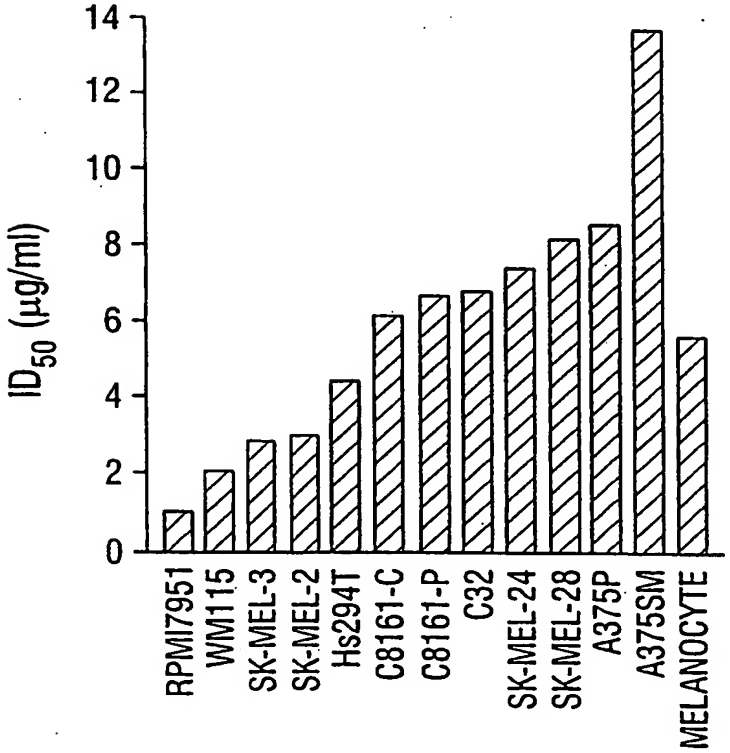

FIG. 3B

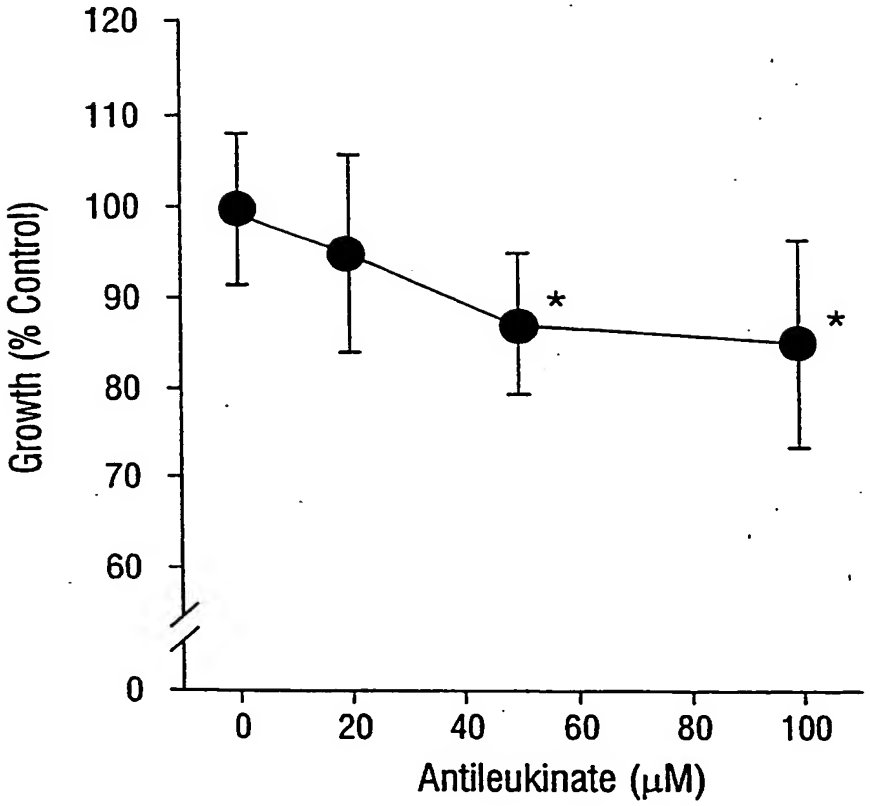

FIG. 4A

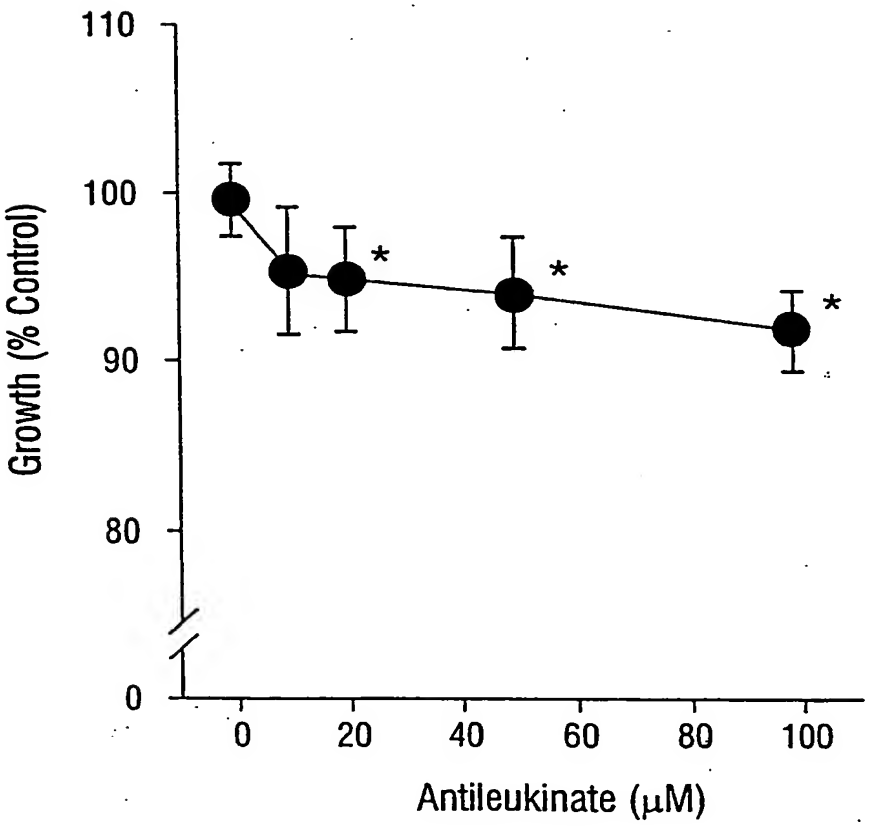

FIG. 4B

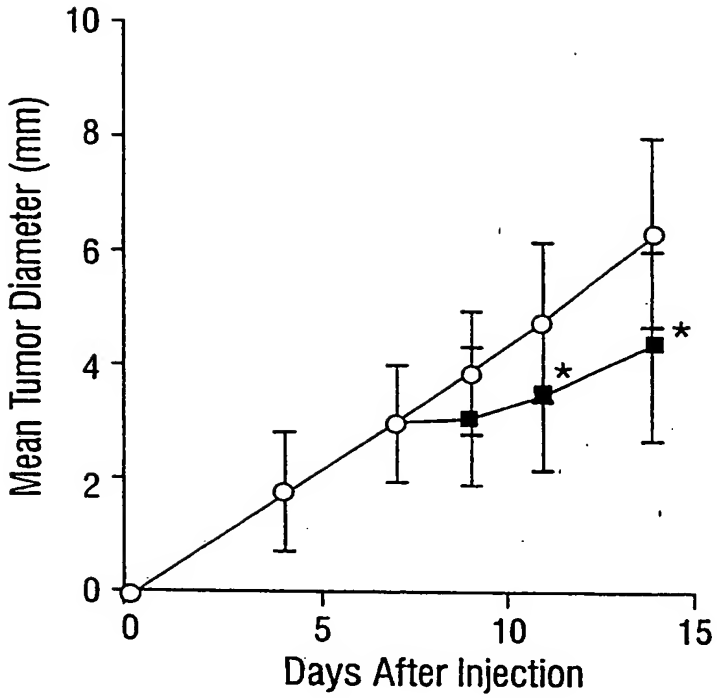

FIG. 5

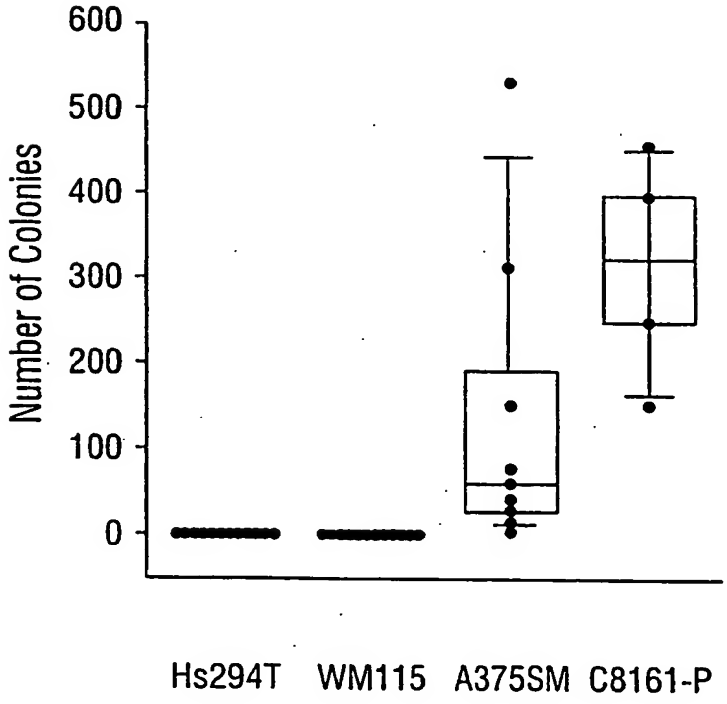

FIG. 6

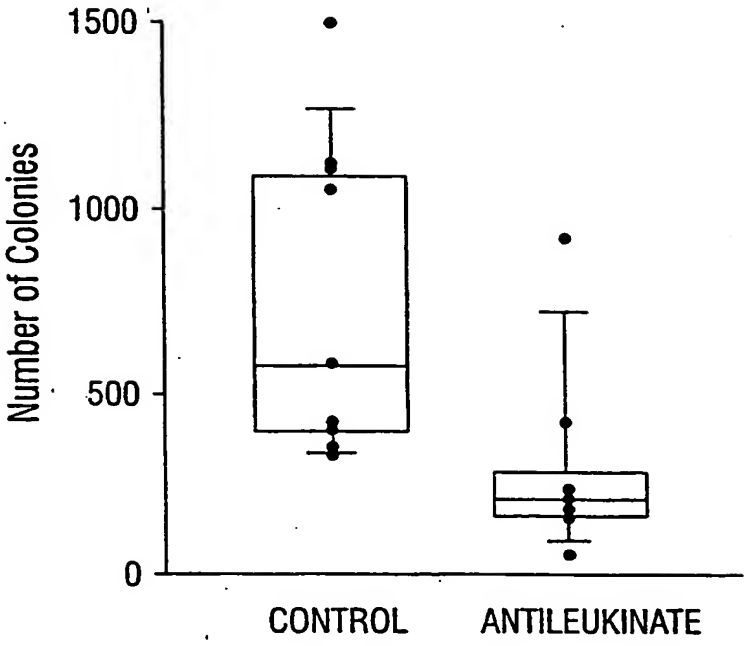

FIG. 7

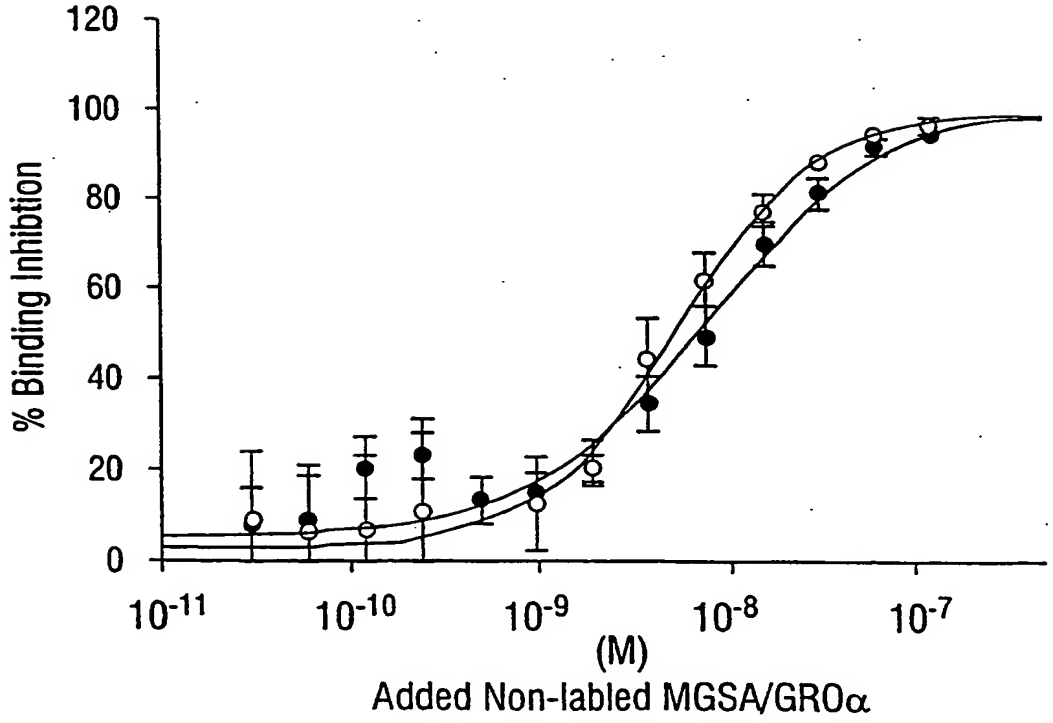

FIG. 8A

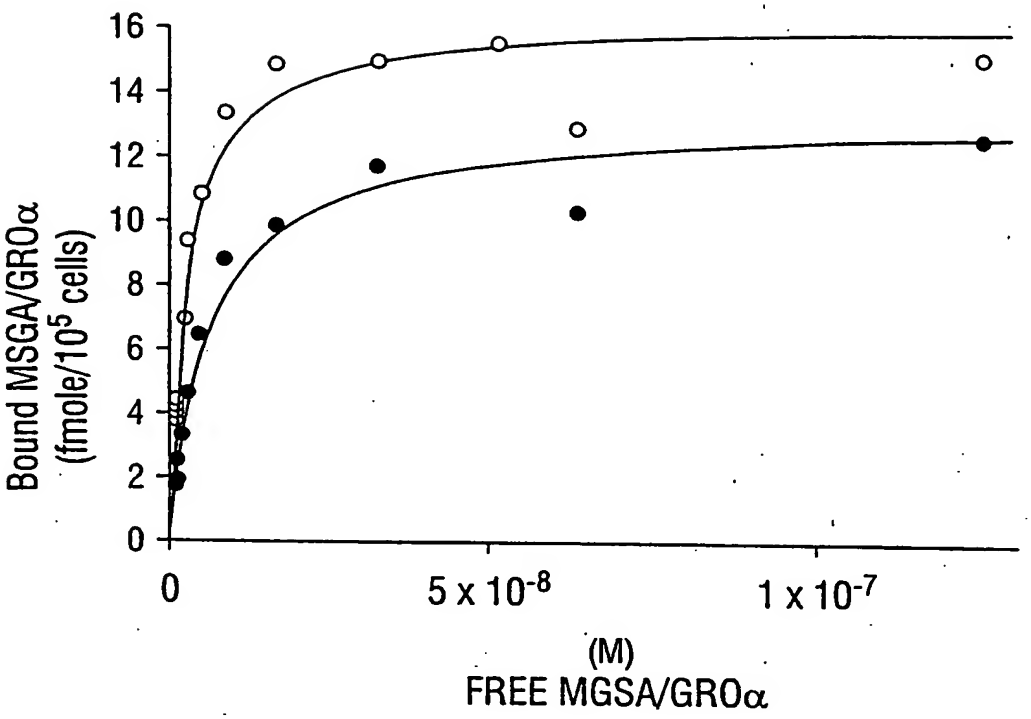

FIG. 8B

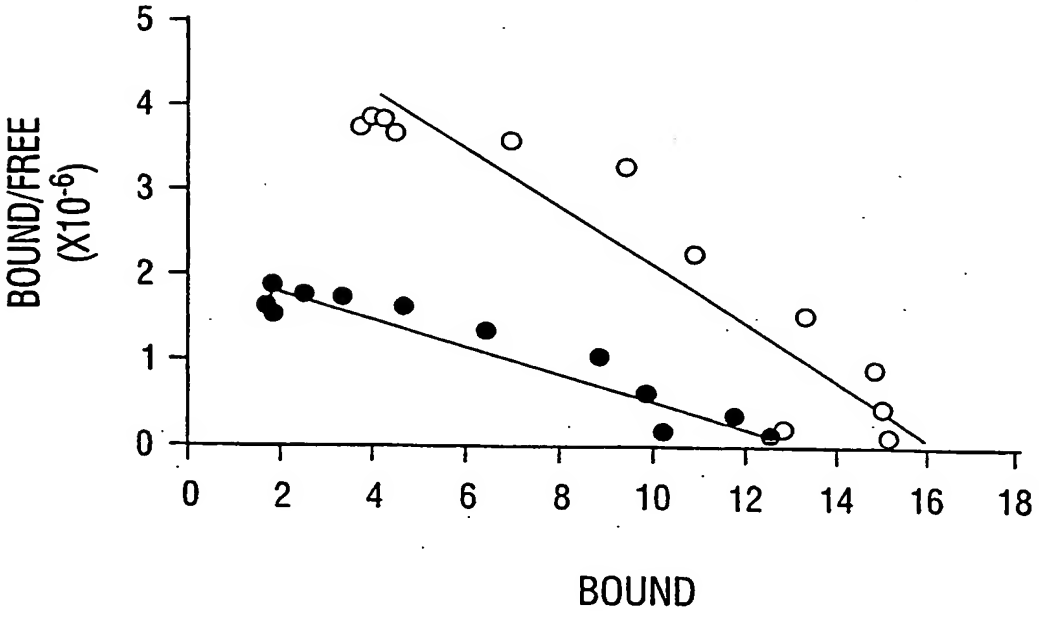

FIG. 8C

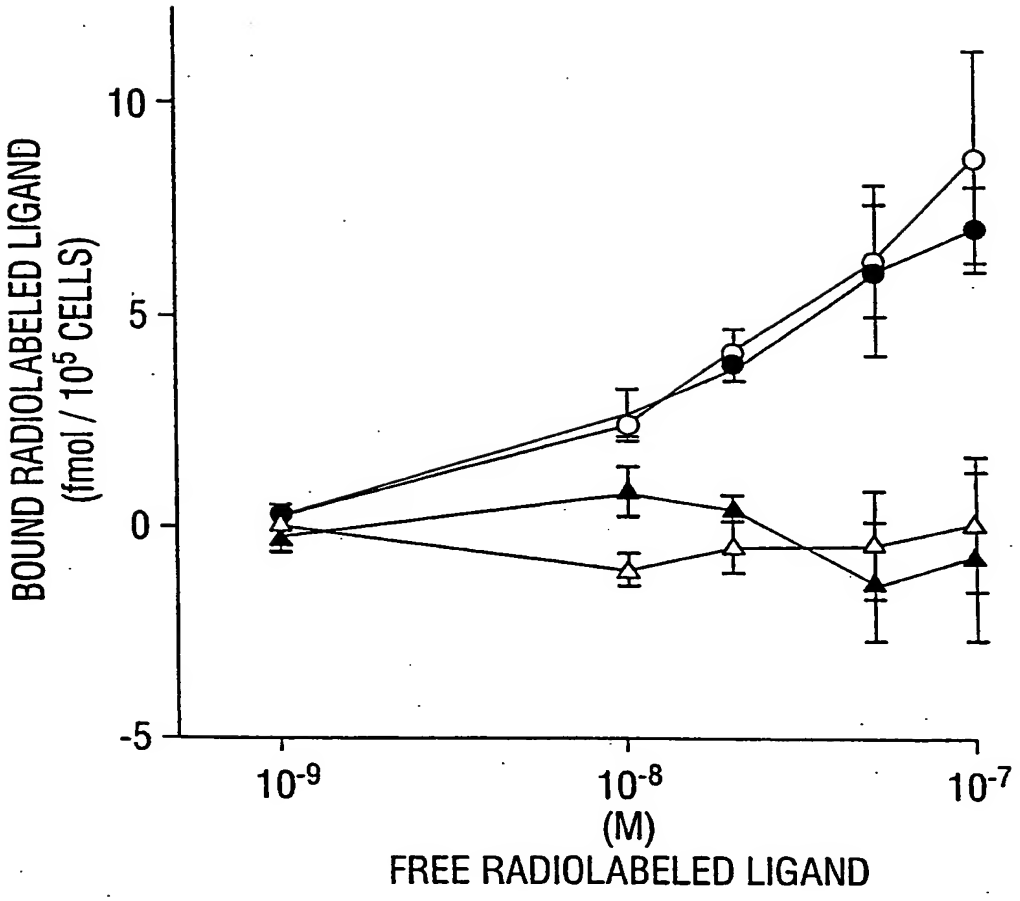

FIG. 9

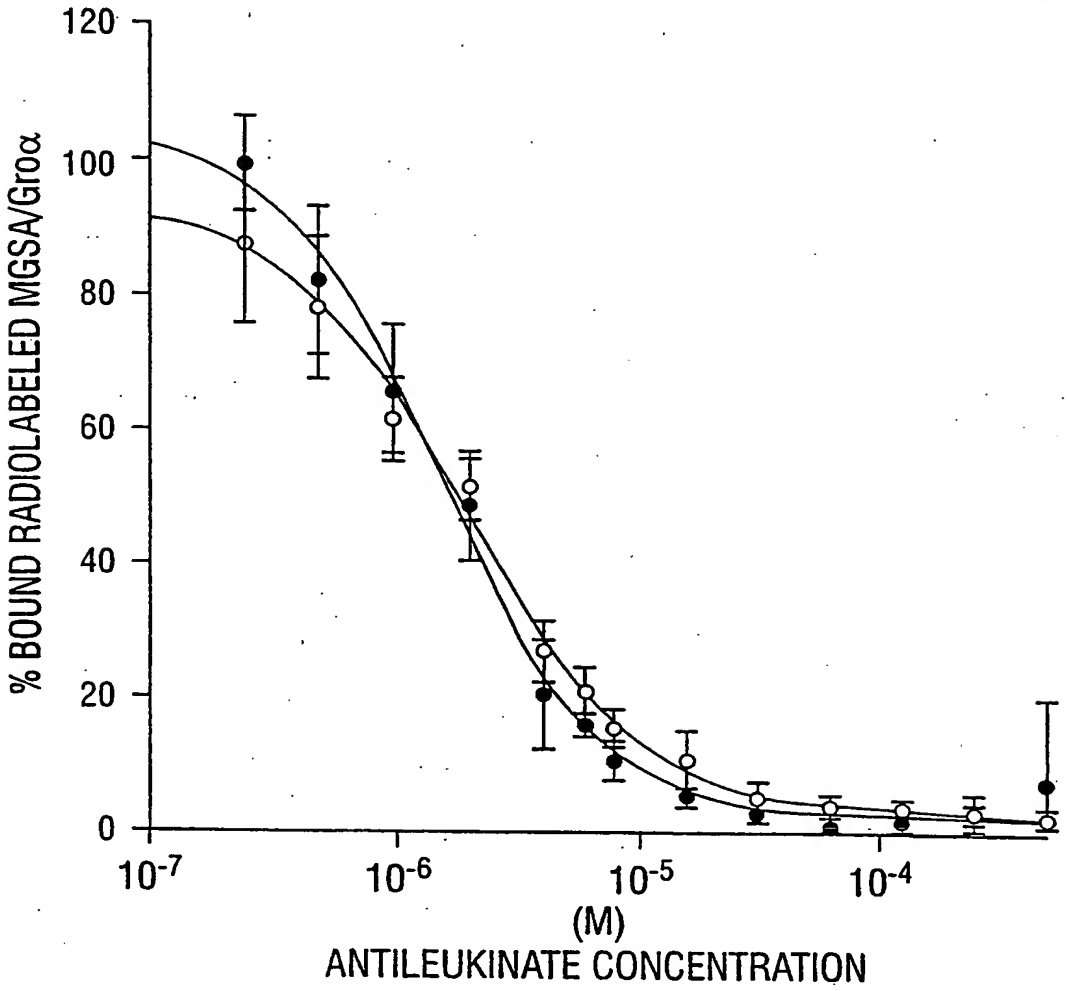

FIG. 10

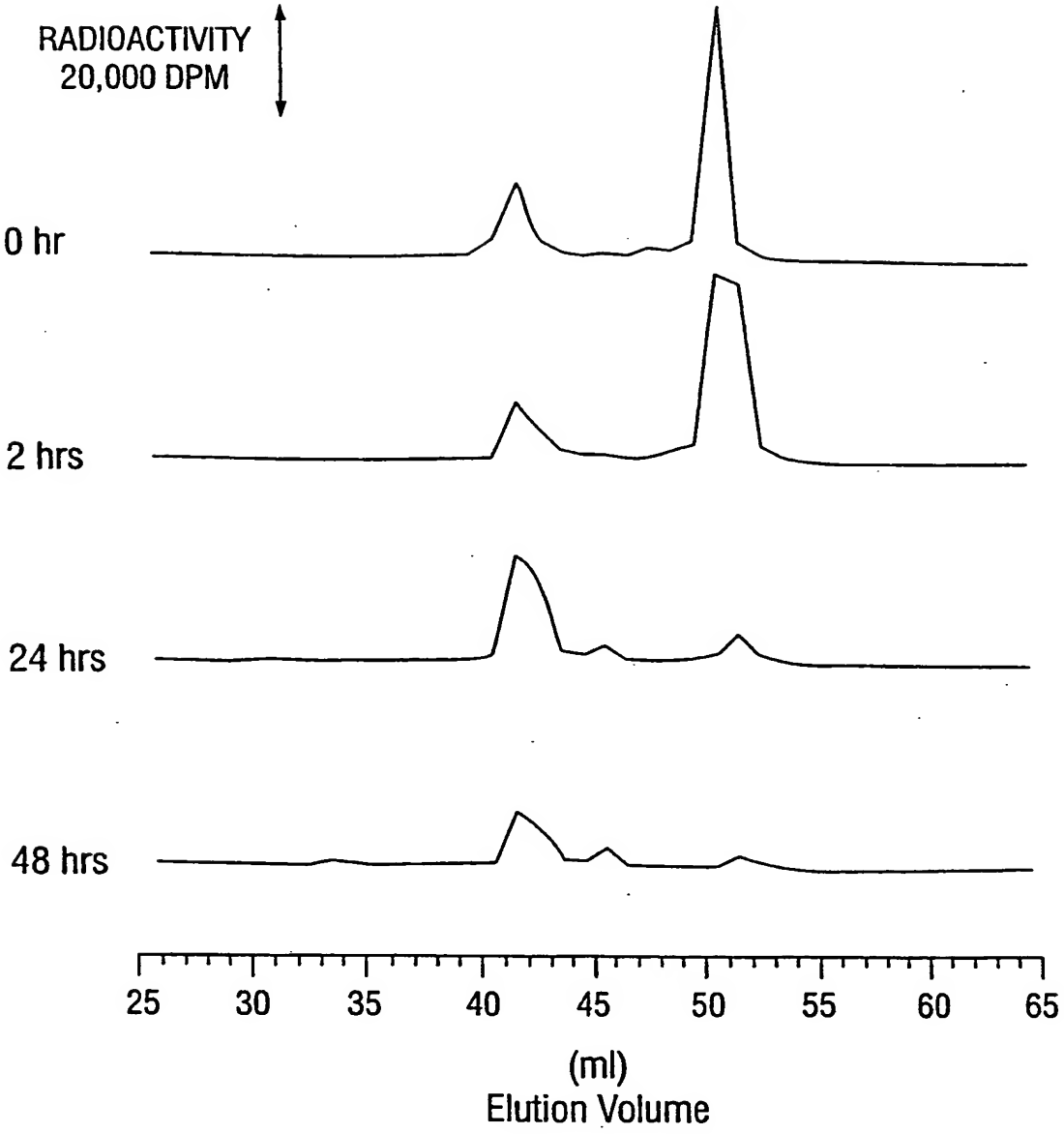

FIG. 11A

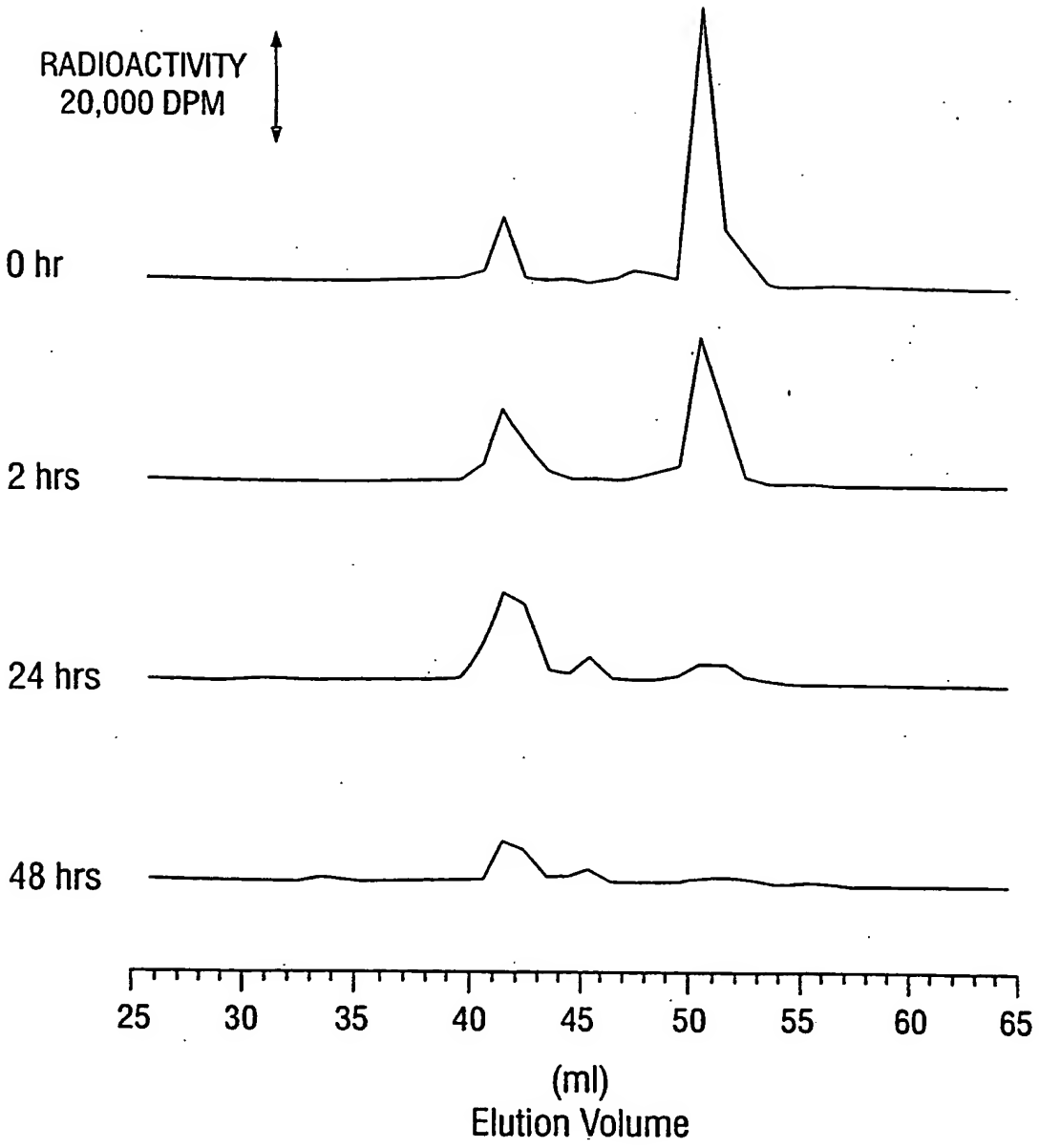

FIG. 11B

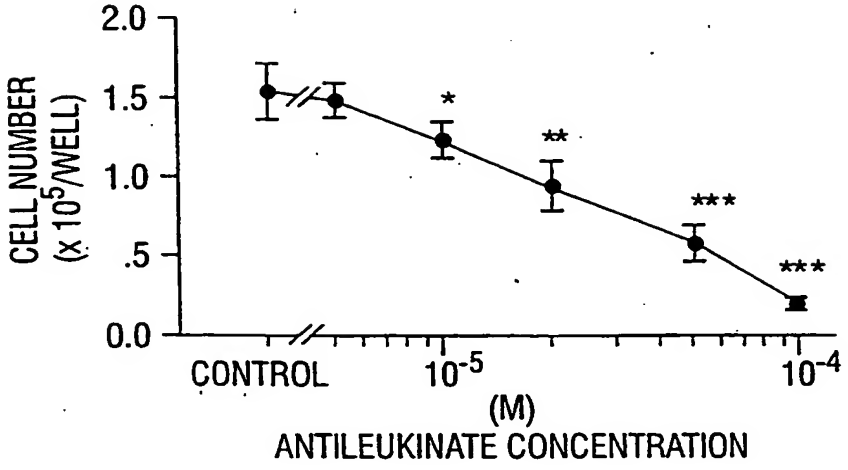

FIG. 12A

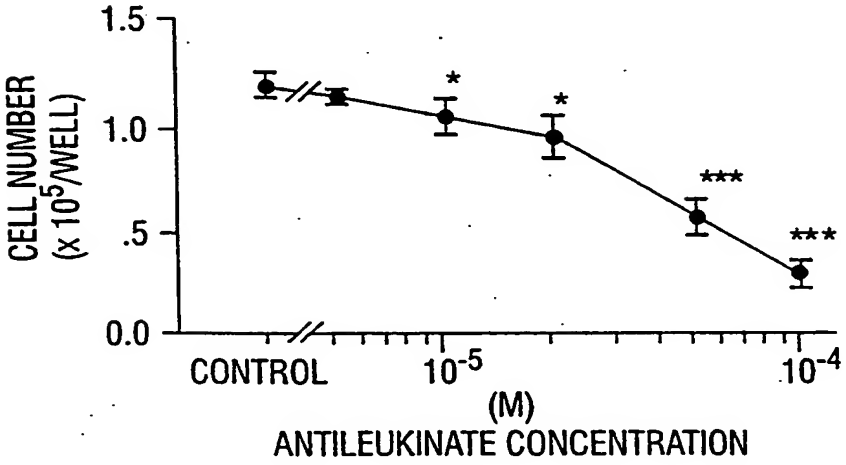

FIG. 12B

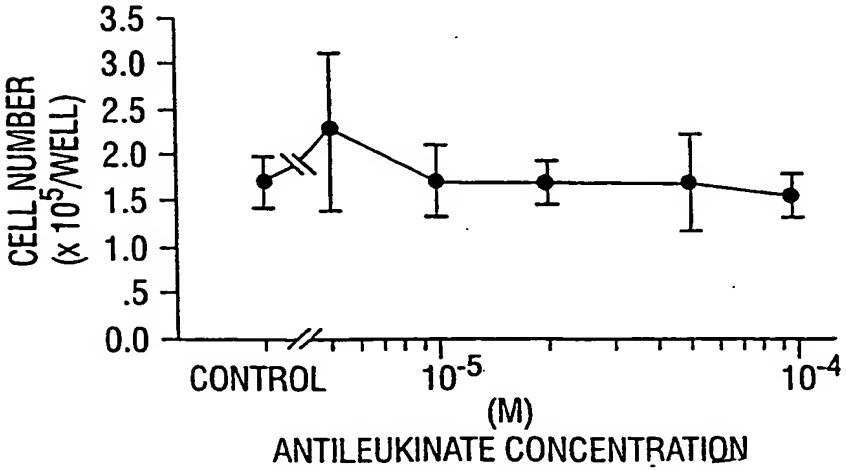

FIG. 12C

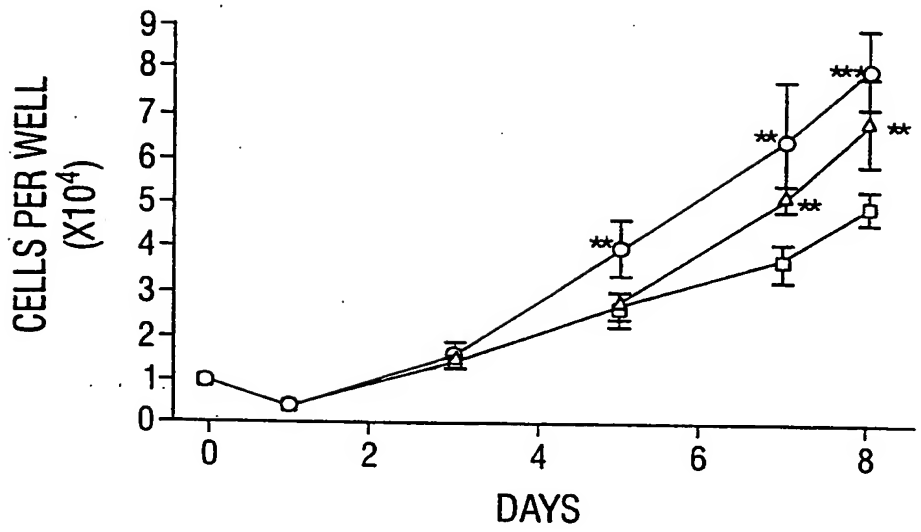

FIG. 13A

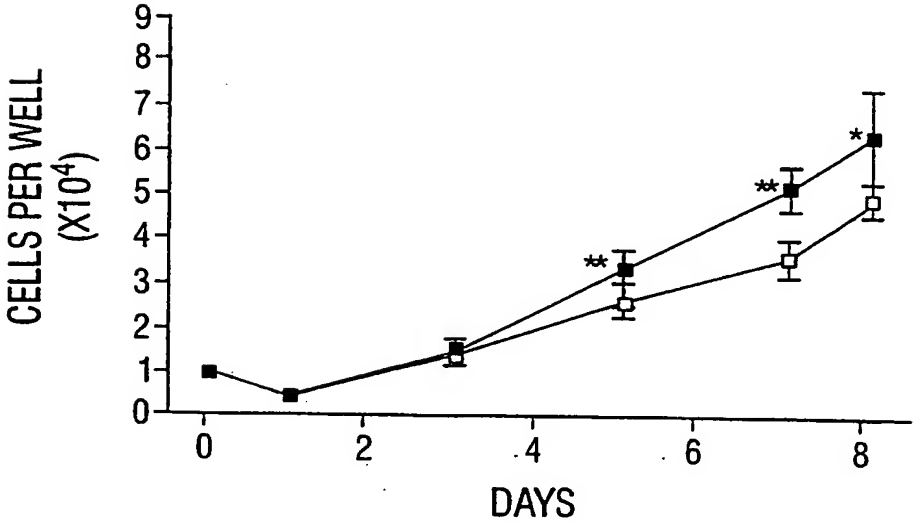

FIG. 13B

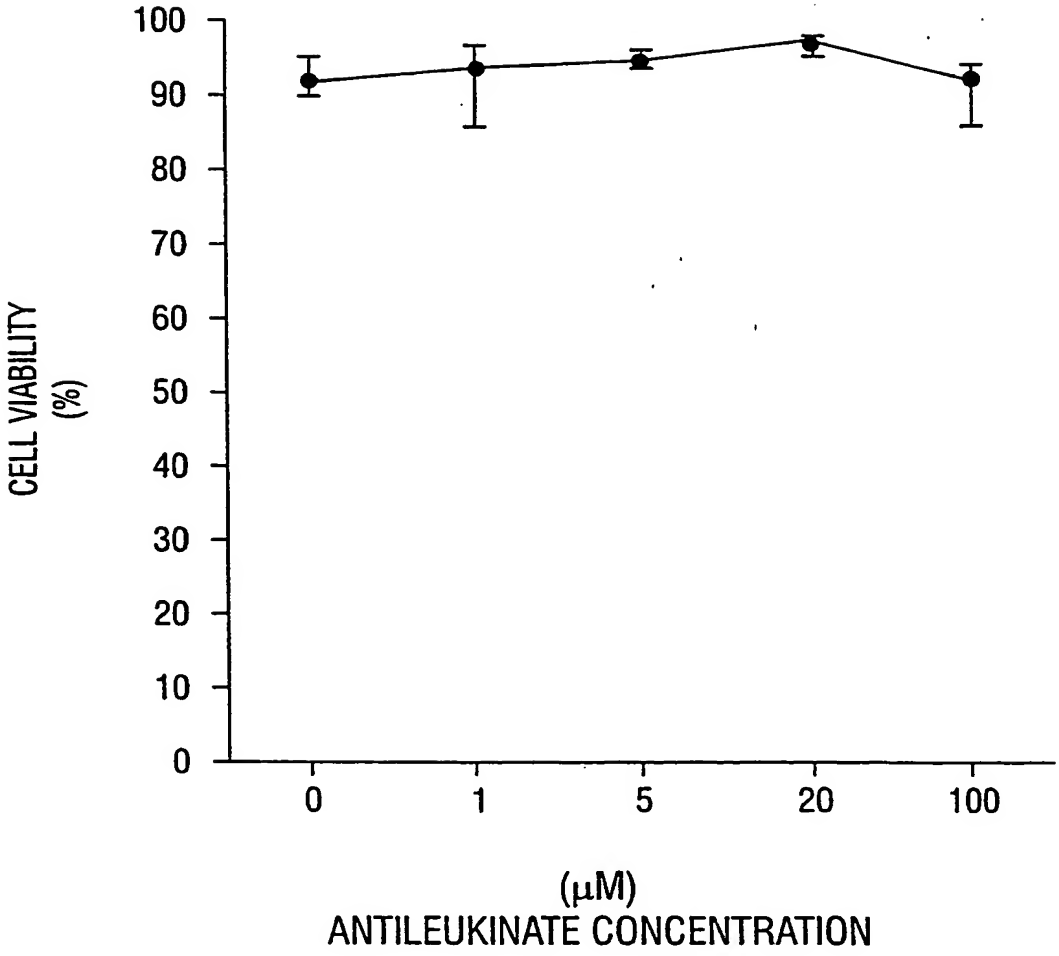

FIG. 14

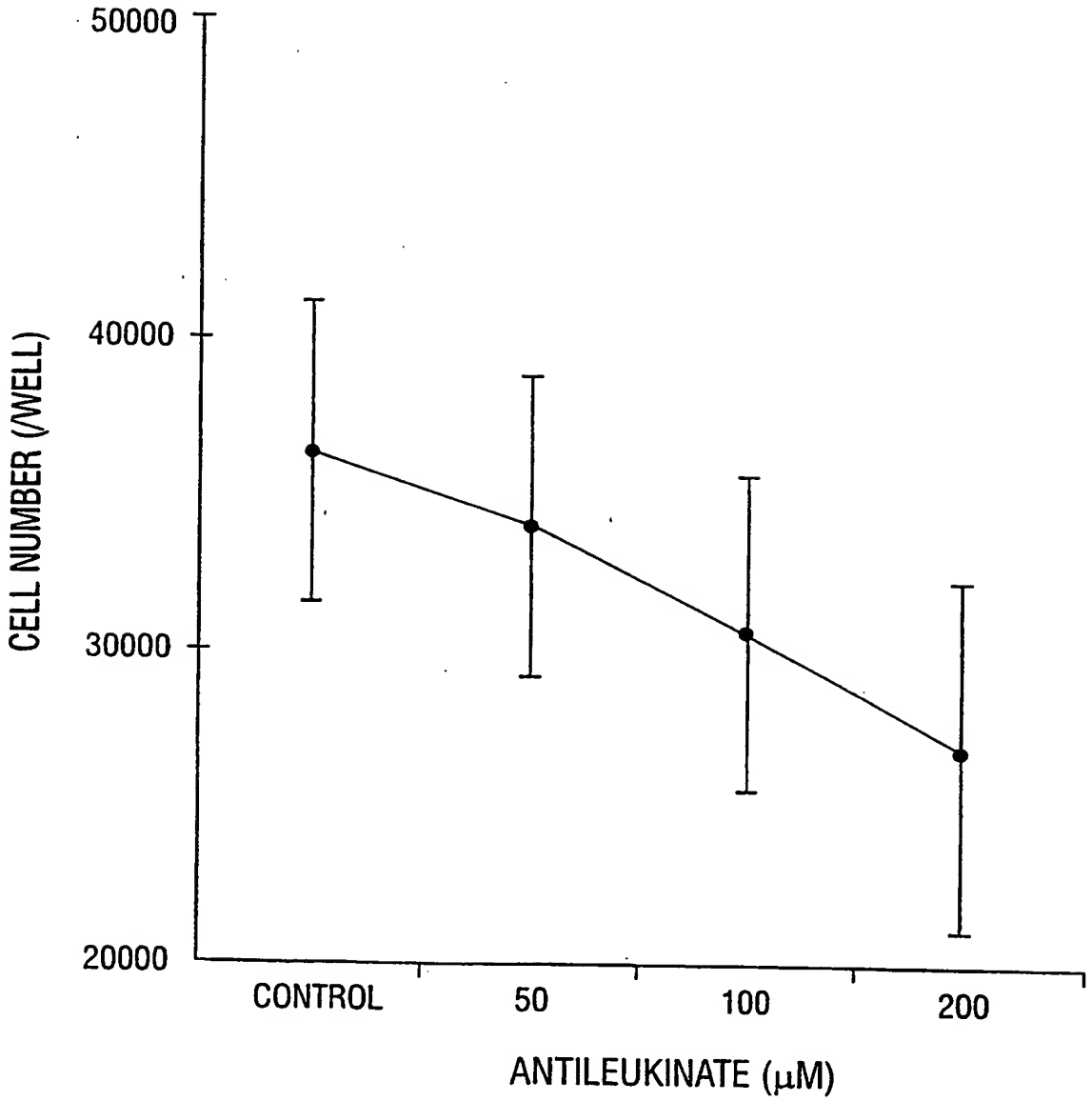

FIG. 15

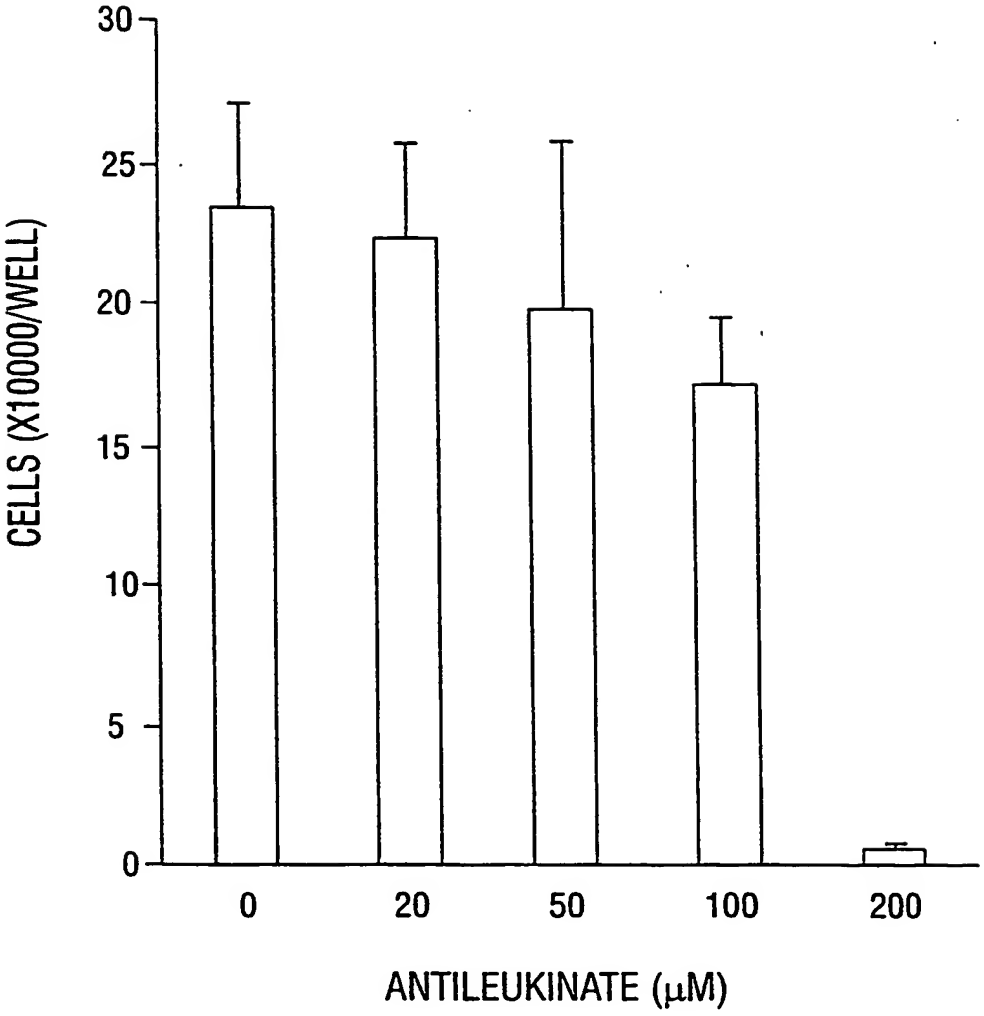

FIG. 16

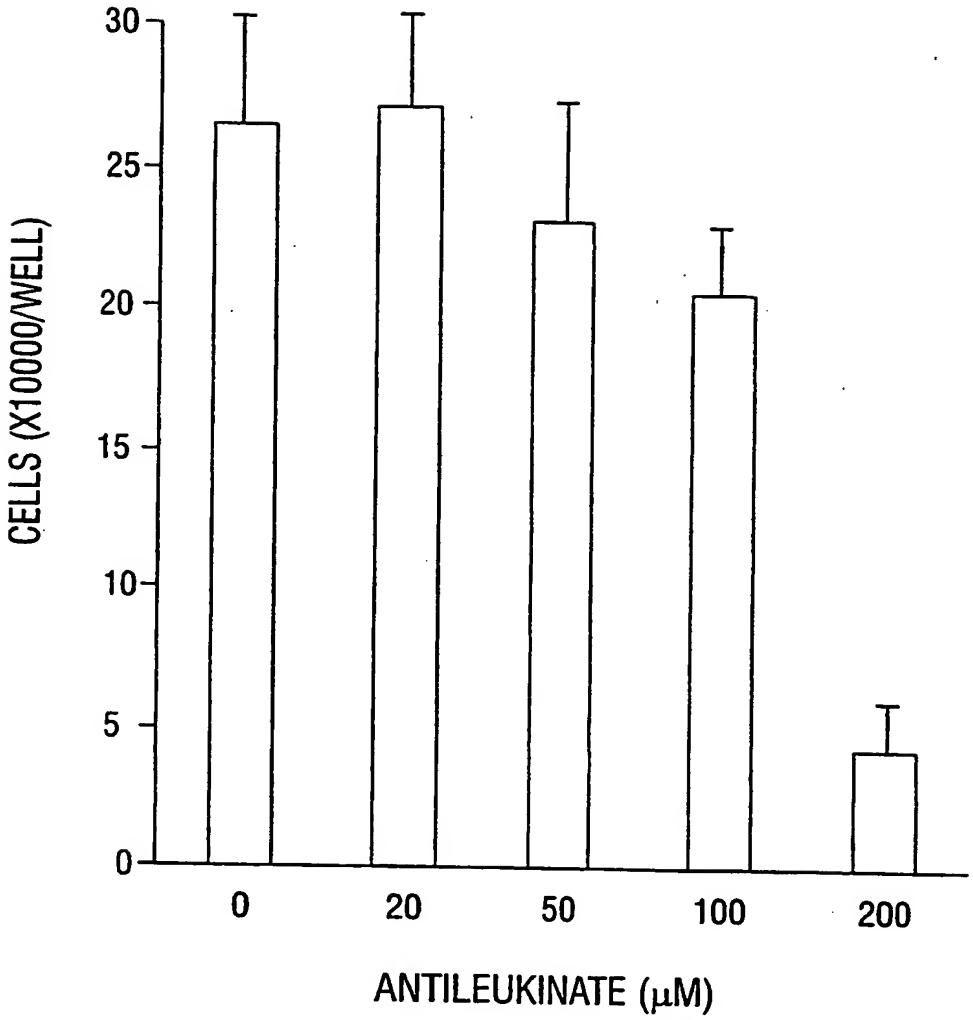

FIG. 17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/10896

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C07K 7/06; A61K 38/08, 38/10
US CL : 514/16, 17
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/16, 17

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN ON LINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MILLER, E. J. et al. Peptide inhibitor of interleukin-8 (IL-8) reduces staphylococcal enterotoxin-A (SEA) induced neutrophil trafficking to the lung. Inflamm Res. 1996. Vol. 45, pages 393-397, see entire document.	1-2
X	HAYASHI S. et al. Synthetic hexa- and heptapeptides that inhibit IL-8 from binding to and activating human blood neutrophils. J. Immunol. 1995, Vol. 154, pages 814-824, see entire document.	1-2
X	WO 95/16702 A1 (BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM) 22 June 1995, claims 1-62.	1-4

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
14 AUGUST 1998

Date of mailing of the international search report
14 OCT 1998

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
MICHAEL BORIN
Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/10896

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/10896

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

Group I, claims 1-4, drawn to method for inhibiting binding of alpha-chemokine to neutrophils.

Group II, claims 5-22, drawn to method for inhibiting binding of alpha-chemokine to tumor cells, inhibiting proliferation of a melanoma cell and inhibiting tumor growth.

The inventions listed as Groups do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: peptide comprising the amino acid sequence of SEQ ID No: 23 is the technical feature that links Groups I and II. Such peptide is not the contribution over the prior art because it is suggested by references teaching peptides comprising the identified sequence. For example, such peptides are taught in WO 95/16702 which demonstrates their use in inhibition of IL-8 binding to neutrophils.